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ROMANOWSKY STAINING WITH BUFFERED SOLUTIONS, III. EXTENSION OF THE METHOD TO ROMANOWSKY STAINS IN GENERAL

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ABSTRACT.—**Solutions at 0.3 g. per 100 cc. of equal parts of glycerin and methyl alcohol of various Wright, Giemsa, Leishman and Balch stains and similar eosinates of thiazene dyes give satisfactory wholesale staining of sections without differentiation when buffered with citric-acid and sodium-phosphate. Prestaining with alum hematoxylin adds to depth, density and permanence of nuclear staining, but decreases clarity. A satisfactory modification of Mayer's acid hemalum is described. The reaction should be pH 4.2 for neutral formalin or Orth fixation, pH 4.6 for acid formalin, pH 5.0 for Zenker formalin and pH 6.5 for ethyl or methyl alcohol or Carnoy fixation. Toluidine blue phloxinate is found to be a quite desirable stain and its preparation is described. Clarite and clarite X are definitely superior to neutral Canada balsam, and somewhat inferior in regard to fading compared with liquid petrolatum as mounting media for these Romanowsky stains.**

Since the earlier publications (Lillie and Pasternack, 1932, 1936) this general technic has undergone some minor modifications which have tended to make it easier to use and to improve the keeping quality of the preparations. Recently a fairly extensive test of various commercial and experimental Romanowsky stains has been completed, which tends to widen the usefulness of the method. The purpose of this paper is to report these changes and experiments.

The basic purpose of the method is to adjust the reaction of the staining mixture so that no subsequent differentiation is required, and in fact should be avoided. This enables us to stain large numbers of sections simultaneously with consistent results, and to repeat the staining procedure precisely from day to day.

Apparently any of the compounds or mixtures of eosin and related

dyes with thionin, toluidine blue, the azures and methylene blue may be used. The following were actually tried in a recent test, the results of which are presented in Table 1: Balch stain (lab. lot 1939), Wright's stain (CWr-12, NWr-10, and a pooled lot of surplus Army supply left over from 1918), Leishman's stain (Grübler), Giemsa stain (Grübler, Cummings, Biosol, Coleman and Bell 06–28–36, and NGe-3), thionin eosinate (Hartman Leddon 1938) and toluidine blue phloxinate (lab. lot 5–8–35).

The preparation of our laboratory Balch stain has already been reported in detail (1936). Toluidine blue phloxinate is prepared by precipitating toluidine blue with phloxine in aqueous solution in the approximate proportion of 2 mols (611.3) of toluidine blue to 1 mol (829.5) of phloxine B (C. I. No. 778) or 1 mol (758.5) of phloxine (C. I. No. 774). Since a slight excess of toluidine blue is desirable, 10 g. toluidine blue (62% dye content) in 500 cc. of distilled water is added to 10 g. phloxine B (82% dye content) in 500 cc. of distilled water. For phloxine, and for dyes of higher or lower dye content, the amounts are adjusted accordingly. After standing overnight, the precipitate is collected on hard filter paper with a suction filter and dried. The filtrate should be a very pale clear blue, indicating practical exhaustion of both dyes from the mixed solutions.

At first (1932) 1 g. eosinate of polychrome methylene blue to each 100 cc. of glycerin and methyl alcohol was used, but experience showed that there was a considerable residue of undissolved dye and the quantity was later (1936) reduced to 0.6 g. Further trial showed that 0.25 g. dye precipitate left no residue and 0.3 g. a slight residue. Comparison of the stain solutions thus prepared with the previous stock in which 0.6 g. had been used showed no appreciable difference in staining.

The Balch stain, the various Wright and dry Giemsa stain samples, the Leishman stain, the thionin eosinate and the toluidine blue phloxinate were dissolved at the rate of 0.3 g. in 50 cc. of C.P., neutral, anhydrous glycerin by standing overnight at 56°C. The glycerin solutions were then cooled and 50 cc. C.P. neutral methyl alcohol added. These constituted the stock solutions. The Giemsa stain solutions were used as furnished.

The buffer solutions are those previously described (1936) and consist of M/15 citric acid in 50% methyl alcohol and M/15 K_2HPO_4 or Na_2HPO_4 in distilled water. (See Table 2.) For the former sufficient accuracy may be attained by weighing out 14.01 g. monohydrated crystalline citric acid, dissolving it in 500 cc. distilled water and adding sufficient C. P. neutral methyl alcohol to bring

TABLE 1. STAINING EFFECT OF 12 ROMANOWSKY STAIN SAMPLES WITH AND WITHOUT PRELIMINARY HEMATOXYLIN STAINING AT VARIOUS pH-LEVELS

	pH	Wright CWr-12	Wright NWr-10	Wright war stock	Balch Lab 1939	Leishman Grübler	Thionin eosinate	Toluidin blue phloxinate	Giemsa solution Biosol	Giemsa solution Grübler	Giemsa solution *Cummings	Giemsa (dry) C & B 06–28–36	Giemsa (dry) NGe-3
Hematoxylin prestain	4.2	++	+	+++	+++	+++	+++	+++	+++	+++	+	+++	+++
	4.6	+++	+++	++	+++	+++	+++	++	+++	+++	±	+++	++
	5.0	+++	+	+	+	±	+	+	+	+	±	++	±
No prestain	4.2	++	+	+++	++	++	+++	++	+++	+++	++	++	+++
	4.6	+++	+++	++	+++	+++	+++	+++	+++	+++	±	+++	+++
	5.0	+++	+	++	+	+	++	+	++	+	±	+++	+

+++ = Excellent
++ = Very good
+ = Good
± = Fair

*This dye gave +++ staining with and without hematoxylin at pH 3.9.

the total volume up to 1,000 cc. after thoro mixing. For the phosphate solution, 9.47 g. C. P. anhydrous Na_2HPO_4 or 11.62 g. C. P. anhydrous K_2HPO_4 is weighed out, using the specially purified salt designed for buffers. This is dissolved in distilled water. Recent experience in the south indicates that it would be well to use 25% methyl alcohol as the solvent for both buffer solutions. This prevents the deterioration due to the growth of molds.

TABLE 2. REACTIONS OF VARIOUS MIXTURES OF M/15 CITRIC ACID AND M/15 DISODIUM PHOSPHATE

cc. M/15 citric acid	cc. M/15 Na_2HPO_4	pH	cc. M/15 citric acid	cc. M/15 Na_2HPO_4	pH
1.3	0.7	3.9(3.873)	0.9	1.1	5.4(5.428)
1.25	0.75	4.0(4.034)	0.85	1.15	5.7(5.696)
1.2	0.8	4.2(4.205)	0.8	1.2	5.85(5.838)
1.15	0.85	4.4(4.44)	0.75	1.25	6.05(6.036)
1.1	0.9	4.6(4.653)	0.7	1.3	6.3(6.29)
1.05	0.95	4.8(4.80)	0.65	1.35	6.5(6.444)
1.0	1.0	5.0(5.042)	0.6	1.4	6.5(6.522)
0.95	1.05	5.2(5.201)	0.55	1.45	6.6(6.60)

These figures are derived from Stitt's variation (1923) of McIlvaine's series.

When a fresh batch of buffer solutions is prepared, they are customarily checked by staining several sections at 0.2 pH variations in the expected range and selecting the proportion giving the best staining. This remains quite constant for the batch of buffer solutions.

The staining technic is as follows:

Stock stain solution as above	2 cc.
Acetone, C. P	3 cc.
Methyl alcohol, C. P	3 cc.
Buffer solution to give the desired pH-value	2 cc.
Distilled water	30 cc.
	40 cc.

Bring paraffin sections to water as usual. Stain 1 hour in the foregoing mixture; rinse in water; dehydrate in acetone; pass thru a mixture of equal parts of acetone and xylene; clear in 2 changes of xylene.

Material fixed in formalin or Orth's fluid stains best at pH 4.2–4.6 (1.1 to 1.2 cc. M/15 citric acid and 0.8 to 0.9 cc. M/15 disodium phosphate); acid formalin material and slightly autolyzed autopsy material fixed in neutral formalin stain better at pH 4.6 or 4.8; sublimate formalin and Helly's or Maximow's formalin Zenker material are best at about pH 5.0 and material fixed in methyl alcohol, absolute alcohol or Carnoy at about pH 6.5. Decalcified material may stain better at 0.4 pH higher than similar undecalcified material.

The various stains used all give quite comparable and usually quite similar results. Toluidine blue phloxinate gives a particularly agreeable picture. The metachromasy of mast cell granules is perhaps more pronounced than with any of the polychrome methylene blue or azure eosinates, thus rendering them more easily distinguishable from somatic or bacterial chromatin. The deeper red staining of erythrocytes and eosinophil leucocyte granules is also quite desirable.

The proper pH-level for staining with any given batch of dye is best determined by staining a number of sections from the same block at 3 or 4 pH-levels varying by about 0.2 in the expected range for the fixation. This level will remain quite constant for the dye sample over a number of months at least.

Increased sharpness, density and permanence of nuclear staining may be attained by staining 3–5 minutes in an alum hematoxylin, washing thoroly and blueing in tap water (with 0.1 to 1% sodium acetate if necessary) before placing in the buffered Romanowsky stain mixture. Delafield's or Harris' formulae are quite satisfactory. A modified Mayer's acid hemalum, quickly prepared and quite stable, has been found very satisfactory. This is prepared as follows:

Dissolve 5 g. hematoxylin (certified) in 700 cc. distilled water, add 1 g. sodium iodate and 50 g. aluminum ammonium sulphate (ammonia alum). Dissolve. Let stand over night, then add 300 cc. C. P. neutral glycerin and 20 cc. glacial acetic acid. It is ready for immediate use.

Prestaining with hematoxylin, however, somewhat diminishes the clarity and brilliance of the stain. Sections prestained in hematoxylin may be mounted in neutral balsam; but without the hematoxylin and in daylight or under an electric light, sections fade fairly rapidly in neutral balsam as compared with sections mounted in heavy liquid petrolatum. The latter is undoubtedly the best preserving medium for this type of stain, but it is messy, it often leaks even with pyroxylin seals, and its index of refraction is rather low ($N_D = 1.483$). This low index of refraction is sometimes advantageous. Recently

trials have been made of clarite and clarite X as mounting media for these stains. So far the preparations have shown much less fading than have similar preparations mounted simultaneously in neutral Canada balsam and exposed to daylight or to an incandescent tungsten light under identical conditions. There has been slight fading as compared with sections mounted in the heavy mineral oil. No appreciable difference between these two new resins is yet evident after a month under continuous exposure to a 100 watt tungsten light at 20–25 cm. distance. The fact that these resins set hard is an obvious advantage over mineral oil.

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IMPROVED PARAFFIN SCHEDULES FOR PLANT TISSUES

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The two paraffin schedules here presented have produced less distortion in plant tissues than those commonly used. Both are modifications of the schedule described by Hemenway.[1] Schedule A is very similar to that described by Hewitt.[2] Schedule B usually causes less distortion than A but staining is not so bright as after A.

Schedule A

(1) Fix tissues 48 hours in Karpechenko, Flemming Strong (half strength)[3] or other solutions that have been found most satisfactory for the tissues under investigation.

(2) Wash tissues 6 hours in running water.

(3) Place tissues on paraffin oven in an evaporating dish filled with the following solution: 100 cc. of 10% glycerin to which has been added 1 cc. of a 10% solution of thymol in 95% alcohol. (Put a canopy about 6 inches above the dish to allow air circulation but to prevent dust from accumulating in it.)

(4) If tissues settle on the sides of the dish during evaporation of water, slide them toward the center of the dish so that they will remain immersed in the concentrating glycerin.

(5) After all of the water has evaporated (10 to 13 days) transfer tissues to normal butyl alcohol and glycerin, 1:3, for 24 hours.

(6) Normal butyl alcohol and glycerin, 1:1, 24 hours.

(7) Normal butyl alcohol and glycerin, 3:1, 24 hours.

(8) Pure normal butyl alcohol on oven, 24 hours.

(9) Transfer tissues to new normal butyl alcohol in new vial and use new cork to eliminate film of glycerin present on first vial and cork. Leave on oven 24 hours.

(10) New normal butyl alcohol on oven, 2 changes, 48 hours in each. (Agitate vial very gently twice daily while tissues are in butyl alcohol to hasten the removal of glycerin.)

(11) Add new butyl alcohol and place solid blocks of paraffin on a

[1]Hemenway, A. F. 1930. Some new methods and combinations in plant microtechnique. Science, 72, 251–2.

[2]Hewitt, W. B. 1938. Leaf-scar infection in relation to the olive knot disease. Hilgardia, 12, 41–65.

[3]Rawlins, T. E. 1933. Phytopathological and Botanical Research Methods. John Wiley and Sons, Inc., New York.

screen suspended in the upper portion of the butyl alcohol; place open vial in a well ventilated paraffin oven. Remove screen after 24 hours and allow remainder of butyl alcohol to evaporate in oven.

(12) Pour off melted paraffin and add new melted paraffin.

(13) Change melted paraffin 2 more times at 24 hour intervals and imbed after tissues have been in the last melted paraffin for 48 hours.

SCHEDULE B

(1) Follow schedule A thru step 10.

(2) Place tissues in normal butyl alcohol and cedar oil, 3:1, in an open vial and leave on oven until butyl alcohol has evaporated. (Use cedar oil labeled "for clearing".)

(3) Paraffin, 25%, in cedar oil, on oven, 24 hours.

(4) Paraffin, 50%, in cedar oil, in oven, 24 hours.

(5) Paraffin, 75%, in cedar oil, in oven, 24 hours.

(These mixtures of paraffin in cedar oil may be used a number of times before discarding.)

(6) Melted paraffin, 24 hours in oven.

(7) New melted paraffin, 24 hours in oven.

(8) New melted paraffin, 6 days in oven.

(9) Imbed.

A SECTION-SMEAR METHOD FOR PLANT CYTOLOGY[1]

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ABSTRACT.—**An improved method for making root tip preparations of plants with medium or small chromosomes is described. Division figures are oriented in polar view by cutting paraffin cross sections of roots; the pectic substance is removed from the middle lamellae and the sections then are smeared. The procedure recommended is as follows: Kill root tips in Navashin or other fluid containing chromic acid. Wash, dehydrate, embed in paraffin, and section transversely. Stain slides bearing the sections by a Feulgen technic following the modification of De Tomasi, except that the hydrolysis is prolonged to 45 minutes, and a 10-minute washing in running water is introduced between the fuchsin and the first bath in sulfite solution. Dehydrate and mount in thin Canada balsam; then apply local pressure to top of cover glass with the tip of a scalpel, while observing the action under low power of a microscope. The prolonged hydrolysis softens the tissue and removes sufficient pectic substance so that cells of the section separate readily and may be flattened until the chromosomes come to lie in a single plane. The slide is permanent.**

Within the past few years root tip smear methods have proved themselves very useful in plant cytology, especially in those species with large chromosomes, such as *Tradescantia*, *Trillium*, *Vicia*, *Allium*, etc. Some difficulties have been experienced, however, in applying these methods to species with medium or small chromosomes. In these latter species, the chromosomes generally lie entirely within the metaphase plate, which is oriented at right angles to the long axis of the cell. When roots of these plants are smeared, the figures usually come to lie on the slide in equatorial view; and the small size of the cells makes it difficult to apply sufficient pressure to turn the figures so that they may be studied in polar view, without severely damaging them.

With the present method, which is largely a modification and combination of technics already in use, the difficulty is solved by applying pressure to paraffin cross sections of roots, and thus combining the proper orientation of sections with the flattening and spreading

[1]This investigation has been supported in part by a grant from the Carnegie Corporation to the Carnegie Institution of Washington.

of smear methods. It was worked out on *Melandrium*, which has medium-sized chromosomes (2–6 μ in length) that are quite numerous in the polyploid forms.

The following is an outline of the section-smear schedule: Roots are killed and fixed 12–24 hours in Navashin or other suitable fluid containing chromic acid. They are washed, dehydrated and embedded in rubber-parowax,[2] using one of the newer dehydrating agents, such as *n*-butyl alcohol. When embedding, it is well to arrange roots from a plant in groups, all roots within a group being parallel and with tips even. Cross sections of roots (in the groups) are cut sufficiently thick to include whole division figures, but not so thick as to be two nuclei deep; 10 μ sections are about right for *Melandrium*. The sections of the embryonic regions of all of the roots in a group usually can be placed on a single slide. After drying the slides, the paraffin is removed in the usual manner and the sections are run down a graded series of alcohols to water. The slides are washed one-half hour or longer in running water (overnight washing does no harm and often removes the last traces of chromic acid stain from the tissues).

The Feulgen method[3] is used for staining, except that slides should be hydrolized for 45 minutes instead of the shorter periods usually recommended. In addition, the slides should be washed in running tap water for 10 minutes upon removal from the leuco basic fuchsin and then passed thru three changes of sulfite solution, allowing 3 to 10 minutes in each change. After passing up an alcohol series to xylene, the slides are mounted in thin Canada balsam, using large, No. 1 cover glasses. The viscosity of the balsam is important in spreading the cells; a little experimenting will quickly show the proper dilution to use.

After the slides have partially dried (preferably 3 to 15 days after mounting) a figure with chromosomes well spread may be located, and while viewing this figure under the microscope (a 10× ocular and 20× objective are convenient), local pressure is applied to the top of the cover glass with the side of the tip of a scalpel. The scalpel is held in one hand, and the microscope is focused with the other, thus keeping the figure and the scalpel tip constantly in view. (A mechanical stage to hold the slide in a fixed position is almost a necessity.) The pressure serves to separate the cells and is best applied in the form of several firm strokes, just to one side of the section containing the division figure. The pectic substance of the middle

[2]Hance, R. T. 1933. A new paraffin embedding mixture. Science, **77**, 353.
[3]De Tomasi, J. A. 1936. Improving the technic of the Feulgen stain. Stain Techn., **11**, 137–44.

lamellae largely dissolves out by the prolonged hydrolysis, and the repeated scalpel strokes should cause the cells to separate and float free in the balsam. Releasing the pressure rapidly at the end of a stroke often facilitates separation of the cells. Pressure should not be too great and should not be applied directly over a section, or the cover glass may break. With a little practice, the cell containing the desired division figure can be coaxed out undamaged from its original position in the section, with few, if any, adhering cells. One should not attempt to flatten the cell in its original position in the section; it must be isolated, comparatively free from other cells.

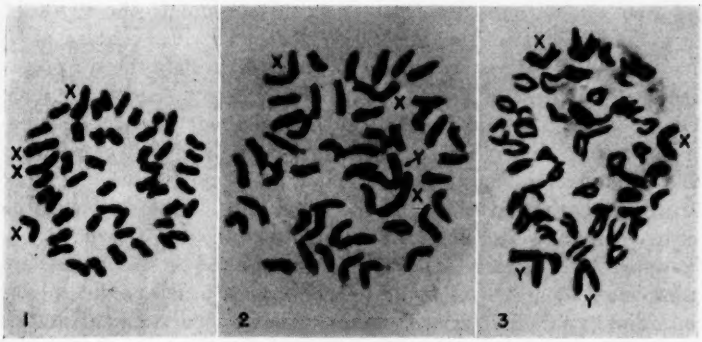

Fig. 1–3. Photomicrographs of section-smear preparations of *Melandrium* root tips, × 1400: Fig. 1. Tetraploid female (4A + XXXX). Fig. 2. Tetraploid male (4A + XXXY). Fig. 3. Tetraploid male of the type 4A + XXYY. The chromosomes lie in one plane and are well separated. Note that sex chromosomes can be distinguished from autosomes, and that varying degrees of chromosome splitting and chromatid separation are shown.

Sufficient pressure is now applied (in the form of one or more strokes directly over the isolated cell) to flatten it and thus bring the chromosomes to lie in one plane (Fig. 1–3). The cytoplasm is soft after the hydrolysis, and gentle pressure is sufficient. If the pressure is released gradually after this flattening, the cell adheres to the slide and remains fixed in this position. The chromosomes now are ready for study. Or, if the first figure does not prove satisfactory, more figures may be located and the flattening process repeated. Figures may be studied before, during, and after flattening, and generally very little rearrangement or distortion of the chromosomes occurs. The preparations are permanent, being mounted in balsam, and will last almost indefinitely.

It is essential that a killing and fixing fluid be used which contains

chromic acid. Hillary[4] has shown that hydrolysis may be lengthened to nearly an hour with no essential reduction in staining intensity after chromic acid fixation, but that 8 minutes' time is the maximum that can be employed without damage after fluids containing no chromic acid.

The 10-minute wash in running water immediately after the basic fuchsin is not included in the usual Feulgen schedule, and apparently is not necessary under some conditions. It greatly increases the intensity of the color reaction in *Melandrium*, however, and is essential to good, sharp contrast. When the basic fuchsin solution becomes colored because of repeated use, it may be regenerated by the addition of a few crystals of potassium metabisulphite ($K_2 S_2O_5$).

The application of this method to *Melandrium* has enabled us to identify the sex chromosomes and to count accurately the autosomes in large numbers of plants.[5] Figures 1–3 show the chromosome constitutions of tetraploid plants of three different types: XXXX female, XXXY male, and XXYY male, respectively. These figures show the chromosomes split and preparing for anaphase separation. In figure 2, the separation has occurred only in the region of the centromere, and was not visible until after the cell was flattened. In figure 3, the process has gone further; many of the chromatids are completely separate. A figure in this stage is usually unintelligible in ordinary paraffin sections, because the two separating chromatids lie in different planes. By this method, however, they are flattened and brought to lie side by side, where they can easily be studied.

The present technic is not recommended for root tip studies on species with large chromosomes; with species having medium or small chromosomes, however, it has proved to be more rapid, in the end, than the straight smear methods, because almost every slide provides a determination. It has been used almost exclusively on somatic tissue, but preliminary tests on sections of anthers and ovules indicate that it also may be useful in flattening and spreading meiotic division figures.

[4]Hillary, B. B. 1939. Use of the Feulgen reaction in cytology. I. Effect of fixatives on the reaction. Botan. Gazette, **101**, 276–300.

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STAINING BACTERIA AND YEASTS WITH ACID DYES

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ABSTRACT.—**Various acid dyes prove satisfactory for the routine staining of bacteria. Those used are acid fuchsin, anilin blue w. s., fast acid blue R, fast green FCF, light green, orseilline BB, erythrosin, phloxine and rose bengal. Acid fuchsin, fast green, anilin blue, and orseilline are especially recommended. Phenolic solutions of the dyes, acidified with acetic acid, with the addition of ferric chloride to those containing acid fuchsin, anilin blue, fast green or light green, are used. Procedures are given in detail for staining or demonstrating vegetative cells, resting and germinating spores, capsules, sheaths and glycogen in bacteria; germinating and conjugating spores of yeast; and for counterstaining after acid fast or Gram staining. The principal advantages of using acid dyes are better differentiation, and less tendency for slime amd debris to take the dye.**

INTRODUCTION

Comparatively little use has been made of acid dyes for staining bacteria. Conn and Holmes (1926) state: "It had been assumed in the past that bacterial stains must be basic dyes; and the use of the acid dyes of this group (fluorescein) had never apparently occurred to bacteriologists." In the last edition of "Biological Stains" (Conn, et al, 1940) one finds only a few references to the use of acid dyes for staining bacteria. Those mentioned are mainly fluorescein dyes, but include also brilliant yellow as counterstain for acid fast bacteria, fast acid blue R for bacteria in soil, light green and eosin in Wright's stain for capsules and spores. During the past three years the writer has used several acid dyes for routine staining of bacteria, and to some extent in classes in general bacteriology. The results have been so satisfactory that it seems desirable to publish an account of the uses, formulas of solutions, staining procedures and advantages of these dyes.

Conn (1918) reported using a 1% solution of rose bengal in 5% aqueous phenol for staining bacteria in soil. Three years later (1921) he emphasized the fact that rose bengal has great affinity for bacterial protoplasm, but not for slime or debris with which the cells may be surrounded; and also that it is unusually well adapted for revealing structure in bacterial cells. He proposed that it be employed as a general bacterial stain. Winogradsky (1924, p. 140)

used erythrosin for soil bacteria. Conn and Holmes (1926) and Conn (1926) investigated erythrosin, rose bengal B and eleven other dyes in the fluorescein group. They listed phloxines, erythrosin Y, and rose bengal as good for staining bacteria. Some improvement in technic was also suggested. A little later Conn and Holmes (1928) recommended the addition of a trace of a salt of Ca, Al, Mg or Pb, especially $CaCl_2$, to staining solutions of eosin, erythrosin, phloxine and rose bengal. Romell (1934) published a procedure for staining soil microorganisms with a phenolic solution of fast acid blue R.

It seems, therefore, that of the acid dyes only certain of the fluoresceins have been used to any extent for staining bacteria, and mainly soil organisms. On the basis of my own experience several other acid dyes can be recommended for general staining of bacteria, especially acid fuchsin, fast green FCF, acid fuchsin and fast green mixed, anilin blue w. s., and orseilline BB.

Some advantages of using acid dyes for staining bacteria may be mentioned. The staining solutions listed below are easy to prepare and apparently will keep several months without deterioration. No fixing with heat is needed. Staining is very rapid, particularly with acid fuchsin, fast green and anilin blue w. s. As a general rule slime and debris stain lightly. Slides stained with acid dyes are never messy and require less washing than is usual with basic dyes. It is possible to secure marked differentiation within the vegetative cell or between spore and parent cell, by using one dye as a primary stain and a second one to destain the first and to act as a counterstain at the same time. It seems also that not only rose bengal, as stated by Conn (1921), but most of the acid dyes used here are well adapted for revealing bacterial cell structure. Apparently the staining is quite permanent with anilin blue w. s., fast green or acid fuchsin used singly or mixed, fast acid blue R and orseilline BB. Slides of large spirals in a mixed culture stained with fast green in 1937 show no evidence of fading; and others of about the same age stained with other dyes are still deeply colored. The writer has not tested the fluorescein dyes for permanency.

The following dyes were used in this work: acid fuchsin, Coleman and Bell, uncertified; anilin blue w. s., Cert. No. NK3; erythrosin, Cert. No. CEr-3; fast acid blue R, National Aniline and Chemical Company; fast green FCF, Coleman and Bell, uncertified; light green SF yellowish, Cert. No. CL-3; orseilline BB, Grübler; phloxine, Cert. No. NPh6 and NPh7; rose bengal, Grübler.

The organisms stained included mainly *Alcaligenes viscosus*, *Bacillus cereus*, *Bacillus megatherium*, *Bacillus mycoides*, *Bacillus subtilis*,

Bacterium coli, clostridia in mixed cultures, *Pseudomonas pyocyaneus*, *Rhizobium meliloti*, *Rhizobium trifolii*, *Rhodococcus roseus*, *Saccharomyces ellipsoideus*, *Sarcina lutea*, *Serratia marcescens*, *Spirillum rubrum*, spirochaetes, etc. from the mouth, *Staphylococcus citreus*, and *Streptococcus lactis acidi*.

The pure cultures of bacteria listed have been used in class work for a number of years at the University of Missouri. Staining and physiological reactions indicate that they are correctly identified. So far as is known they were received from the following sources: *Alcaligenes viscosus*, *Bacillus cereus* and *Spirillum rubrum*, American Type Culture Collection; *Bacillus megatherium*, *Bacterium coli*, American Museum of Natural History; *Rhodococcus roseus*, Army Medical Museum; *Streptococcus lactis*, isolated at the University of Missouri. *Rhizobium meliloti* and *Rhizobium trifolii* were obtained directly from fresh nodules on living hosts. The exact source of the other six species of bacteria is unknown. *Saccharomyces ellipsoideus* was received in 1925 and seems to be correctly identified.

Formulae

Solution A. Phenol, 5% aqueous.....30 cc.
Glacial acetic acid, 20% aqueous.....8–10 cc.
Ferric chloride, 30% aqueous..... 4 cc.

Add a 1% aqueous solution of any one of the following dyes in the quantities indicated: acid fuchsin (1–2 cc.); anilin blue w. s. (2–8 cc.); fast green FCF (2–8 cc.); light green (2–8 cc.); fast acid blue R (15 cc.) omitting ferric chloride; orseilline BB (8 cc.) omitting ferric chloride.

Solution B. Phenol, 5% aqueous.....100 cc.

To solution B add 1 gram of rose bengal, erythrosin or phloxine, previously dissolved in about 5 cc. of distilled water. Then add drop by drop enough (6–8 drops) 20% aqueous acetic acid to cause a trace of cloudiness. Finally add 20 to 50 cc. of 95% alcohol. This seems to improve the solution and if a slight cloudiness has developed it should disappear; if not add a trace of ammonium hydroxide in 95% alcohol.

Mixed solutions of dyes. Acid fuchsin (solution A plus dye) may be added to either fast green (A plus dye) or light green (A plus dye) in the proportion of 1 part acid fuchsin to 4 parts of the other; or enough acid fuchsin may be added to the green dye to produce a deep reddish purple color. This mixture stains a greenish blue to nearly purple. It has considerable selective action too, especially if applied for only a few seconds. Spores, for example, may stain light pink and the mother cells green to purple.

In general aqueous or phenolic solutions of acid fuchsin, anilin blue, fast green, or light green stain bacteria very lightly or scarcely at all. The use of the ferric chloride is therefore very important but the part it plays is unknown.

Staining Procedures

In the staining procedures described here it will be understood that whenever a particular dye is mentioned it has been made up according to one of the formulae given above.

Vegetative cells. In general the procedure for staining vegetative cells of bacteria with acid dyes is to prepare a smear in a drop of water, dry without heating, stain 10 seconds to 1 minute, wash with water and dry. A longer time (2–3 minutes) is better with the fluorescein dyes and with fast acid blue R. Staining is very good and clear-cut with fast green, acid fuchsin, anilin blue w. s., fast green and acid fuchsin mixed, or with acid fuchsin followed by fast green (about 5 seconds). The last procedure gives results similar to those with acid fuchsin and fast green mixed, but by varying the time properly one may obtain more differentiation. This differentiation may frequently be improved by treating 1 minute with Gram's iodine and washing.

If fast acid blue R, orseilline BB, rose bengal, phloxine or erythrosin is used, staining is lighter than with the dyes listed above, but still very satisfactory for most purposes. Staining with these dyes is usually decidedly improved if the bacterial films are first treated 2–3 minutes with solution A, washed with water and then stained. Orseilline BB or rose bengal is to be preferred to phloxine or erythrosin. A *very rapid* (1–2 seconds) counterstain with fast green following the red dyes sometimes brings about considerable differentiation.

One of these dyes may be used to destain another and counterstain at the same time. Fast green, for example, will destain the fluorescein dyes and orseilline very rapidly, and acid fuchsin more slowly. Rose bengal or orseilline will destain fast green, anilin blue w. s., or fast acid blue R in 15 seconds to 1 minute.

The bacteria in nodules of legumes are surrounded by considerable slime. Stained preparations much superior to those made with basic dyes may be obtained by using acid dyes as follows: Make a smear directly from a crushed nodule with or without water. After drying, stain 10–30 seconds with acid fuchsin, wash, stain with fast green 3 seconds and wash. Another satisfactory procedure is to treat 2–3 minutes with solution A, wash, stain 1–2 minutes with orseilline, wash, counterstain with fast green 2 seconds and wash. Using either of these procedures the bacteria will be stained red and the background (slime) green with a clear zone around each cell. For class work material from nodules of either red clover or sweet clover proves to be excellent.

In acid-fast staining, methylene blue is commonly used as a counter-

stain. In class work acid fuchsin and fast green mixed, or else fast green alone has been used for counterstaining. This gives a very sharp contrast since the acid fast organisms appear red and others green.

If carbol fuchsin is used as a counterstain when one uses Hucker's or other procedures for Gram staining, it may withdraw considerable of the gentian violet or crystal violet from Gram positive bacteria, even in 3–5 seconds. This destaining action may be avoided by counterstaining with rose bengal, phloxine or erythrosin, even when staining is prolonged as much as a minute.

Spores. Spores of bacteria may be demonstrated very well with acid dyes. Young spores will stain deeply but mature or old ones only lightly. The cells containing spores, however, can always be stained by the following technic to give a sharp contrast: Treat smears 3 minutes with solution A; wash with water, stain 3–5 minutes with acid fuchsin or fast green, singly or mixed, orseilline or rose bengal; wash with water and counterstain with fast green 2–4 seconds after orseilline, 1–2 seconds after rose bengal, and 5–8 seconds after acid fuchsin; or after fast green counterstain 10–30 seconds with rose bengal (50% alcoholic) or orseilline.

Spores of *Bacillus cereus*, *B. megatherium*, *B. mycoides* or *B. subtilis*, when stained with acid dyes show well various stages in germination. Transfers are made from old spore-bearing cultures to agar slopes and incubated at room temperature for 3–6 hours. Then smears are made and stained as described for vegetative cells, with acid fuchsin 1–2 minutes followed by fast green 5–10 seconds. When stained in this way cells from spores that have just germinated are red while older cells are stained greenish purple.

Instead of growing the bacteria on agar slopes, drops of melted agar may be placed on clean slides and allowed to harden. Spot inoculations from a spore suspension in water may be made with a loop and the slides incubated in a moist chamber. The agar films are then dried and stained as above, or with fast green 10–30 seconds, or with orseilline or rose bengal 1–2 minutes, washed and dried. It is possibly still better to stain with fast green, wash and counterstain with rose bengal for 30 seconds. This results in young germinating spores staining green and other cells pink to purplish red.

Capsules and Sheaths. Another use of acid dyes is for demonstrating capsules or sheaths of bacteria. Films may be made in the usual way; or if there is little slime present it is best first to spread a *very thin* layer of albumen fixative (Mayer's) on the slide, then to add a drop of water and to make a smear. If albumen fixative is

used, one should treat the preparation after drying with absolute alcohol and dry to coagulate the albumen. In either case staining should be with acid fuchsin, or with acid fuchsin and fast green mixed; or the film may be treated with solution A, washed, and stained with orseilline or rose bengal.

Background. A stained background is often desirable in studying bacteria. One may use negative preparations made with aqueous solutions of nigrosin, nigrosin mixed with acid dyes, or Congo red, etc. In such preparations the bacteria are either not stained or only slightly stained. If a smear is made in a *thin* layer of albumen fixative, dried, treated with absolute alcohol, stained with acid fuchsin for 10–30 seconds, washed, and then counterstained 2–4 seconds with fast green, or with acid fuchsin and fast green mixed, and washed, most of the bacteria are stained red and the background is green. Smears made in a *thin* layer of Congo red may be dried and stained with acid fuchsin in solution A. This results in red cells in a blue background.

Glycogen. If 3 or 4 beans are covered with about 2 inches of water in a preparation dish and allowed to incubate for 2–6 days, many clostridia will be found inside and around the decaying seeds. This is very good material for demonstrating the occurrence of glycogen, as well as stages in spore formation, in bacteria. A smear may be made on a slide, dried, stained very rapidly with orseilline, rose bengal, or fast green, washed, treated with Gram's iodine for 1 minute, washed and dried. The dye stains the part of the cell that is free from glycogen, while the glycogen appears a reddish brown. Depending on age the whole cell, or all but a small part of it at one end, may give the reaction for glycogen. If a young spore is present in the cell only that part between the spore and one end of the cell will give the glycogen test.

Yeasts. The mature spores of yeasts are easier to stain than those of bacteria, and stain readily with acid dyes. Spores of *Saccharomyces ellipsoideus* develop rather abundantly in cultures grown for a week or more at room temperature and then kept in a refrigerator for several months. Or they will develop in two weeks or less on agar prepared according to the following formula (Maneval, 1924): water, 1000 cc.; Liebig's beef extract, 3 g.; sodium chloride, 5 g.; dextrose, 2.5 g.; agar, 15–20 g. The spores may be stained with acid fuchsin or fast green; or with orseilline or rose bengal after treatment for 3 minutes in solution A. Then they may be washed with water, dried, and a *very thin* layer of 1% aqueous nigrosin applied. Such preparations show the vegetative cells and spores

stained and the asci colorless. If, however, a counterstain is used before applying nigrosin, fast green (2–8 seconds) for the red dyes or rose bengal (20–30 seconds) for the green, the vegetative cells will be stained with the counterstain.

Saccharomyces ellipsoideus is a favorable organism for demonstrating germination and conjugation of spores. If transfers are made from old cultures containing an abundance of spores to potato dextrose agar slopes, and incubated at room temperature for 5–7 hours, many spores will germinate. Or spot inoculations on hardened drops of agar on slides as described above for bacteria, may be made and, after incubation in a moist chamber, treated as follows: stain with acid fuchsin and fast green mixed, or with acid fuchsin followed by fast green, wash, dry, apply a *thin* layer of aqueous nigrosin and dry. The ascospores, as described by Marchand (1913), may swell and develop vegetative cells directly; or they may enlarge and develop beaks which unite forming conjugation tubes, so that when two ascospores have conjugated they appear somewhat like a capital U or heart-shaped. When these resulting zygospores germinate, buds may form anywhere on them but most frequently on the conjugation tube.

Acid dyes have been used for some time in lacto-phenol mounts of various kinds of organisms, especially fungi. It is evident from the staining procedures and results described here that a number of acid dyes may be used also as general bacteriological stains, and that for certain purposes they are to be preferred to basic dyes. Actually it is possible to perform quite satisfactorily most or all of the staining described here with only two acid dyes, acid fuchsin and fast green.

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AMYL ACETATE AS A CLEARING AGENT FOR EMBRYONIC MATERIAL

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ABSTRACT.—**Amyl acetate is soluble in 95% alcohol and hot paraffin and produces no hardening in objects exposed to its action for prolonged periods. It may be advantageously employed as a general clearing agent and is especially recommended for refractory material. The following schedule has proven satisfactory for whole frog embryos and young tadpoles: 45 minutes to 1 hour in 95% alcohol, 24 hours in amyl acetate, rinse in toluene, 15 minutes each in three changes of paraffin, imbed. Material so treated may be sectioned at 5 μ with comparative ease.**

All of the more commonly used clearing agents produce perceptible hardening, even in tissues exposed to their action for the minimum time. This hardening effect is especially objectionable in the case of highly refractory objects such as amphibian embryos which, in the early stages, contain large amounts of yolk. Yolk granules may be rendered hard enough to injure the knife. Furthermore, some clearing fluids (e.g. dioxan) are chemically active and cannot be used after certain fixatives or interfere with subsequent staining reactions.

The use of amyl acetate, suggested by Barron[1] seems to be free from these objections. This fluid is sufficiently soluble in hot paraffin to permit rapid infiltration and is miscible with 95% alcohol. The use of absolute alcohol with attendant shrinkage and hardening is thus avoided. In addition, no hardening whatever is produced by the most prolonged treatment with amyl acetate. Amphibian eggs have been stored in this fluid for over a year without undergoing any changes in sectioning properties. In fact, in desirability as a storage medium, amyl acetate far surpasses the commonly employed 70% alcohol which may produce noticeable hardening in a week's time.

The writer has found the following procedure to be entirely satisfactory for the treatment of whole frog embryos from the earliest stages thru the late tailbud stage: Following fixation, the embryos are rinsed in water and then transferred directly to 70% alcohol. After about an hour in this solution, the vitelline membranes, if still present, are removed and the embryos transferred directly to 95% alcohol and allowed to remain in this fluid for from 45 minutes to 1 hour—no longer. They are then transferred to pure amyl acetate where they may remain indefinitely, altho a 24-hour treatment (perhaps less) is ample. Each of the alcohols and the amyl acetate should be changed at least once. The amyl acetate is then

[1]Barron, D. H. 1934. Amyl acetate: A useful solvent for embedding masses. Anat. Rec., 59, 1–3.

decanted, the vial containing the embryos is filled with toluene, and the embryos are *immediately* transferred to pure melted paraffin by means of a wide-mouthed pipette. The purpose of the toluene is merely to rinse off the excess amyl acetate which, being relatively involatile, would otherwise accumulate in the paraffin bath. Any other volatile liquid would serve just as well. The paraffin should have a melting point of about 58° C. and the temperature of the oven should be such that a part of the paraffin remains unmelted. The tissue should be allowed to remain for 15 minutes in each of 3 changes of paraffin before imbedding. In no case should the embryos be exposed to this temperature for more than 1 hour. No advantage is gained by inserting in this series a bath consisting of a mixture of paraffin and the clearing agent.

When subjected to this treatment, the embryos remain so soft at the time of imbedding that extreme care must be taken not to injure them during the process of orientation. The instrument used in orienting them should be well heated in a flame before immersion in the paraffin. It is probable that the difficulties usually ascribed to "crystallization" of the paraffin are frequently due to the introduction of minute air bubbles into the paraffin by the use of an insufficiently warmed orienting needle. At any rate, if the needle has been heated until it smokes, the block may be allowed to solidify at room temperature without adversely affecting its sectioning properties. The cutting properties of the paraffin may be improved by the addition of a little gum rubber or one of the prepared compounds containing this substance.

During the past year, a number of other workers in this laboratory have used amyl acetate for clearing a variety of tissues following many special as well as general fixatives and preceding a large number of staining technics. In all cases, the results have been highly satisfactory, the form and staining properties of zymogen granules, mitochondria, etc. being unaffected. It seems to be particularly suitable for clearing large objects which would be excessively hardened in the time required for the penetration of absolute alcohol and the usual clearing agents. Since amyl acetate does not produce optical clearing, the time required must be determined empirically as for the alcohols and the paraffin. Since there is no maximum period, however, this is not a difficult procedure.

The odor of amyl acetate may become quite disagreeable after prolonged inhalation of the vapor. Possible adverse physiological effects have not been noticed but it would probably be wise to observe the same precautions in the handling of this reagent as in the handling of chloroform, dioxan, etc. Waste amyl acetate should be kept in a stoppered bottle, as it is rather difficult to flush down the drain.

ALIZARIN RED S AND TOLUIDINE BLUE FOR DIFFERENTIATING ADULT OR EMBRYONIC BONE AND CARTILAGE

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Abstract.—**This technic has been successfully employed by the author for staining, *in toto*, the bones and cartilage of mature specimens of *Urodela* and the developing bone and cartilage of the embryonic human, cat, pig and rat. The differential staining is accomplished by using a modification of Dawson's method of staining bone with alizarin red S following a toluidine blue solution specific for cartilage. Specimens are fixed in 10% formalin, stained one week in a solution of .25 g. of toluidine blue in 100 cc. of 70% alcohol, macerated 5 to 7 days in a 2% KOH solution, counterstained for 24 hours in a 0.001% solution of alizarin red S in 2% aqueous KOH, dehydrated in cellosolve and cleared in methyl salicylate. In the adult and embryonic forms thus treated the soft tissues are cleared while the osseous tissue is stained red, the cartilage blue.**

Introduction

A number of authors have described procedures for staining the bones and clearing the soft tissues of embryos and small animals *in toto*. Among these workers there exists a difference of opinion with regard to the fixative to be employed. Dawson's (1926) original technic indicated the use of 95% alcohol and with this Lipman (1935) agrees. Gray (1929) employs iodine-alcohol followed by absolute alcohol. The present author, however, has found that tissue fixed in 10% formalin for at least one week can be better controlled during subsequent maceration in KOH. The macerative effect of KOH on tissue which has been fixed in alcohol is comparatively rapid, but there is constant danger that such tissue will too quickly reach the point of total maceration. Such destruction rarely occurs in formalin-fixed specimens, nor is a constant check required to prevent it.

The use of methyl salicylate for clearing tissue (first suggested by Spalteholz, 1914) has two slight disadvantages. Tissue so cleared has a tendency to turn brown over a period of years. This may be remedied by transferring the specimen to a fresh lot of the oil and reclearing. Secondly, the tissue will undergo a slight amount of shrinkage; not enough, however, to affect the relations of the skeletal

parts in any appreciable manner. Cumley, Crow and Griffin (1939) have cleared embryos, previously macerated in KOH, in toluol followed by storage in anise oil saturated with naphthalene. Schultze (1897) macerated his specimens in KOH and cleared them in glycerin. Specimens thus cleared are easily dissected, for the tissues remain soft and pliable and undergo no shrinkage. However, glycerin will render the soft tissues only semitransparent.

Procedure

1. *Preparation and fixation.* If the bone and cartilage are to be submitted to detailed microscopic observations the specimen should be completely eviscerated, the contents of the thorax and abdomen being removed thru a small incision in the abdominal wall. This procedure is not necessary for specimens which are to be used as museum preparations. The specimen is washed in plain tap water and immediately fixed in a 10% formalin solution for at least one week.

2. *Cartilage staining.* The specimen is washed for 24 hours in 250 cc. of 70% alcohol to which 10 drops of NH_4OH have been added. It is then stained one week in a toluidine blue solution prepared as follows: to 100 cc. of 70% alcohol add 2 cc. of 0.5% HCl and 0.25 g. of toluidine blue.[1] The mixture is allowed to stand for 24 hours, at the end of which time it is filtered and stored in a tightly corked container. The specimen is next hardened and destained for 72 hours in four changes of 95% alcohol.

3. *Maceration.* The specimen is macerated in several changes of a 2% aqueous solution of KOH, the length of time depending upon the size of the animal. The average time for early mammalian embryos is from 5 to 7 days, or until the bones of the limbs become visible thru the macerated integument. This process is greatly facilitated by exposure to sunlight or ultra-violet rays.

4. *Bone staining.* The specimen is transferred to a fresh solution of 2% KOH to which is added, by drops, a saturated alcoholic solution of alizarin red S,[1] enough to turn the hydroxide a deep wine red. At the end of 24 hours the bones should appear well stained. If the specimen has been insufficiently macerated the soft tissues will appear slightly stained. In this event the specimen may be quickly destained in acid alcohol (1% H_2SO_4 in 95% alcohol).

5. *Dehydration, clearing and storage.* Dehydration is accomplished by running the specimen thru 3 changes of cellosolve[2] of 6 hours each. For small embryos this time may be considerably

[1]Coleman and Bell dyes (not certified) were used in this technic.
[2]Manufactured by the Carbide and Chemicals Corp., New York City, N. Y.

reduced. (The alcohol series may be substituted for this step, 50%, 80% and 90%, followed by 3 changes of benzene.) The best medium for clearing the specimen after dehydration is methyl salicylate (synthetic oil of wintergreen) which possesses essentially the same index of refraction as the tissues to be cleared. From the cellosolve the specimen is transferred to solutions of 25%, 50% and 75% of methyl salicylate in cellosolve, for 24 hours each. This step must be carefully observed in order to prevent undue shrinkage of the tissues. It is then transferred to pure methyl salicylate for permanent storage.

In animals thus treated the soft tissues are rendered transparent while the osseous tissue is stained a deep red, the cartilage a dark blue. In addition, the intensity of the stains serve to indicate the relative amounts of ossification and chondrogenesis which have taken place. The bone or cartilage may be stained separately by omitting step 2 for bone or step 4 for cartilage. Excellent permanent preparations may be made of parts such as limbs, hands or heads by mounting them in Canada balsam on a deep depression slide. The tissues of such preparation are cleared well enough to permit detailed microscopic observations of the gross osseous and cartilaginous structures.

For those who might prefer clearing in glycerin the author suggests the following modification of the technic presented in this paper: Omitting step 5, transfer the specimen directly from the KOH-alizarin solution into a series of 50%, 70% and 80% glycerin solutions for 24 hours each. It may then be stored in pure glycerin where the soft tissues will continue to clear for several days. Should further clearing be desired the specimen is transferred from the glycerin into cellosolve and treated as per step 5.

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THE REACTION OF CERTAIN STAINS WITH BACTERIA[1]

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Abstract.—**In view of the controversy over the contention that staining is the result of physical or chemical affinity between a stain and a bacterial cell, this paper is presented to show some recent data which support the idea that staining is an adsorption exchange process chemical in nature.**

It is a well established fact that when a stain is added to a suspension of bacteria, a certain amount of the stain is removed from solution and concentrated in and about the cell. The forces that attract the stain to the cell and bind it there have been described as both chemical (Stearn and Stearn, 1929, 1930) and physical (Holmes, 1929) in nature.

The present work is an attempt to show that the staining of a bacterial cell occurs as a result of an adsorption exchange process which approaches the magnitude of stoichiometrical proportions. The data accumulated seem to indicate that the reaction is of a chemical nature.

The mechanism of exchange adsorption may be visualized by the use of equations; for example, a suspension of negatively charged bacteria should attract positively charged ions (McCalla, 1940, a, b).

$$(\text{n bases}^{+}) + (\text{B}^{n-}) \rightleftharpoons (\text{n bases}^{+})(\text{B}^{n-})$$

(B represents the bacterial cell with an unknown number of negative ionic valences, n.).

As represented by this equation the attraction of the negatively charged bacterial cell for the positively charged bases should produce a neutral system; that is, the bases should be adsorbed by the cell. If a stain is adsorbed at the same positions as the bases, it should be be possible to replace an adsorbed base when the stain is added. For example, if methylene blue chloride were added to a suspension of bacteria saturated with adsorbed Mg, at any given concentration, it should result in a displacement of the Mg by the stain, according to the following equation:

$$(\tfrac{1}{2}\text{n Mg}^{++})(\text{B}^{n-}) + \text{nS}^{+}\text{Cl}^{-} \rightleftharpoons (\text{nS}^{+}\text{B}^{n-}) + \tfrac{1}{2}\text{n MgCl}_2$$

(where S represents the stain or methylene blue ion)

It should be possible to measure the displaced Mg chemically and show a stoichiometrical relationship between the methylene blue

[1]Contribution No. 197, Department of Bacteriology.

adsorbed and the Mg displaced. This adsorption exchange view may be tested by an additional method. If normal bacteria with different bases in the adsorption sphere are transformed into a system with predominantly adsorbed hydrogen, it should be possible to add methylene blue chloride and replace the adsorbed H^+, according to the following equation:

$$(\text{n bases}^+)\,(B^{n-}) + (nH^+Cl^-) \rightleftarrows (nH^+)\,(B^{n-}) + \text{n bases}^+Cl^-$$

$$(nH^+)\,(B^{n-}) + (nS^+Cl^-) \rightleftarrows (nS^+)\,(B^{n-}) + nH^+Cl^-$$

The replacement of the adsorbed H^+ should result in an increase in H^+ concentration which may be measured.

Experimental

The following stains—methylene blue,[2] medicinal, 88% purity; crystal violet,[3] 88%; basic fuchsin,[3] 91%; safranin 0,[3] 94%; phloxine—were prepared as 0.01 *M* aqueous solutions, except basic fuchsin which was used in an 0.008 *M* concentration. All stock solutions were kept in paraffined flasks. The adsorption displacement of cations by the use of basic stains was determined with five different organisms, *Staphylococcus aureus*, a spore forming bacillus, and three strains of *Escherichia coli*. The cultures were grown on salts agar[4] in large bottles for one to two days at 37°C. At the end of this period the cells were suspended in distilled water and washed free of dissolved materials by repeated use of distilled water and a Sorvall centrifuge. Since in general staining the organisms are usually dead, the cells were killed by heating at a temperature of 100°C. for three to five minutes. Such cells when stained appeared normal.

Magnesium Displacement with Methylene Blue

It was assumed in the preceding discussion that if Mg were held by bacteria in the adsorption sphere, it should be replaced in staining. To insure the presence of adsorbed Mg, *Escherichia coli* was grown on salts agar containing five grams of $MgSO_4$ per liter. *Staphylococcus aureus* cells were prepared by growing in salts agar and then adding a dilute solution of $MgSO_4$ to the washed cells. These cells were allowed to stand for about thirty minutes before washing free of dissolved Mg. To washed aliquots of the suspension, known quantities of 0.01 *M* methylene blue chloride were added and carefully mixed. The cells were then thrown out of suspension by use of the centrifuge. The supernatant liquid from these cells presumably containing the

[2]Certified by the Commission on the Standardization of Biological Stains.

[3]Found satisfactory by the Commission on Standardization of Biological Stains.

[4]The medium contains the following ingredients: potassium citrate, 1.0 g.; diammonium phosphate, 0.5 g.; potassium bicarbonate, 0.5 g.; dipotassium phosphate, 1.5 g.; peptone, 20.0 g.; dextrose, 0.5 g.; agar agar, 25.0 g.; distilled water to make 1000 ml. This medium was adjusted to neutral reaction with NaOH.

displaced Mg and some unadsorbed methylene blue was evaporated and ignited. The Mg was determined by the method of Baver and Bruner (1939). Controls, one with bacterial cells to which no methylene blue was added and another with only methylene blue, were carried along with each determination. The data given in Tables 1 and 2 are representative and show clearly that when methylene blue was adsorbed by *Escherichia coli* and *Staphylococcus aureus* it replaced Mg with which the cells had previously been saturated. Only a trace of Mg was present in the supernatant liquid from the magnesium-saturated bacteria to which no methylene blue was added, and none was present in the meythlene blue.

TABLE 1. THE DISPLACEMENT OF MAGNESIUM FROM *Escherichia coli* WITH METHYLENE BLUE

Trials	Amount of Mg in Methylene Blue	m.e.* Mg replaced/100 g. Bacteria†		m.e. of Methylene Blue adsorbed per 100 g. of Bacteria
		Water+Bacteria	Methylene Blue + Bacteria	
1	0.0	Trace	21.4	22.8
2	0.0	"	38.5	60.8
3	0.0	"	33.3	52.2
4	0.0	"	60.0	64.0
5	0.0	"	30.6	48.9

TABLE 2.—THE DISPLACEMENT OF MAGNESIUM FROM *Staphylococcus aureus* WITH METHYLENE BLUE

Trials	Amount of Mg in Methylene Blue	m.e.* Mg replaced/100 g. Bacteria†		m.e. of Methylene Blue adsorbed per 100 g. of Bacteria
		Water+Bacteria	Methylene Blue +Bacteria	
1	0	Trace	17.3	30.5
2	0	Trace	10.9	24.1

*m.e. refers to milligram equivalents.
†Expressed as weight of cells dried at 105°C.

HYDROGEN REPLACEMENT WITH BASIC STAINS

Stearn and Stearn (1929) definitely showed that when basic stains, adjusted to a certain pH-value, were added to protein solutions of peptone, gelatin and nucleic acids, also adjusted to the same pH-value, they became more acid. Joseph (1940) from his observations on previous studies of pectin concluded that H^+ was adsorbed by this system. The adsorption was determined by the change in pH when sulphuric acid was added to the pectin system.

To determine whether basic stains would replace H^+ adsorbed by bacteria, a sufficiently dilute solution of HCl was added to washed

cells of bacteria to give a pH-value of approximately five and the unadsorbed H-ions were removed by washing. The pH value of the stain was adjusted above that of the bacterial suspension. Increasing amounts of the basic stains were then added to 10 ml. aliquots of bacterial suspension treated with H^+ (H-bacteria) along with sufficient distilled water to make the volume the same in all instances. The increase in H^+ was measured with both glass and quinhydrone electrodes. All data reported were obtained with the glass electrode.

Some of the typical data are reported in Tables 3, 4, and 5. An

TABLE 3. THE DISPLACEMENT OF ADSORBED HYDROGEN WITH CRYSTAL VIOLET AND SAFRANIN O FROM BACILLI

ml. of H-bacteria	ml. of Stain	ml. of Water	pH of H-bacterial Suspension			
			Treated with Crystal Violet		Treated with Safranin O	
			Actual pH	Decrease in pH	Actual pH	Decrease in pH
10.0	0.0	5.00	6.00	0.0	6.00	0.0
0.0	5.0	10.00	6.55	0.0	7.00	0.0
10.0	0.1	4.90	5.68	0.32	5.60	0.40
10.0	0.25	4.75	5.60	0.40	5.55	0.45
10.0	0.50	4.50	5.45	0.55	5.45	0.55
10.0	1.00	4.00	5.35	0.65	5.28	0.72
10.0	2.00	3.00	4.92	1.08	5.00	1.00
10.0	3.00	2.00	4.58	1.42	4.82	1.18
10.0	4.00	1.00	4.38	1.62	4.82	1.18
10.0	5.00	0.00	4.35	1.65	4.82	1.18

TABLE 4. THE DISPLACEMENT OF HYDROGEN FROM *Escherichia coli* WITH METHYLENE BLUE AND CRYSTAL VIOLET

ml. of H-bacteria	ml. of Stain	ml. of Water	pH of H-bacterial Suspension			
			Treated with Methylene Blue		Treated with Crystal Violet	
			Actual pH	Decrease in pH	Actual pH	Decrease in pH
10.0	0.0	5.00	4.90	0.00	5.60	0.00
0.0	5.00	10.00	5.90	0.00	6.20	0.00
10.0	0.10	4.90	4.80	0.10	5.45	0.15
10.0	0.25	4.75	4.55	0.35	5.32	0.28
10.0	0.50	4.50	4.40	0.50	5.13	0.47
10.0	1.00	4.00	4.18	0.72	4.90	0.70
10.0	2.00	3.00	4.10	0.80	4.50	1.10
10.0	3.00	2.00	4.10	0.80	4.55	1.05
10.0	4.00	1.00	4.10	0.80	4.50	1.10
10.0	5.00	0.00	4.10	0.80	4.50	1.10

examination of the data reveals that in every case there was an increase in H^+ when a basic dye combined with a bacterial system. It is also evident that there was a maximum acidity, and that any addition of dye beyond this range did not result in any further increase in acidity.

TABLE 5. THE DISPLACEMENT OF HYDROGEN WITH BASIC FUCHSIN FROM *Staphylococcus aureus* AND *Escherichia coli*

ml. of H-bacteria	ml. of Stain	ml. of Water	pH of H-bacterial Suspension			
			Staph. aureus		*Esch. coli*	
			Actual pH	Decrease in pH	Actual pH	Decrease in pH
10.0	0.00	5.00	6.00	0.00	4.50	0.00
0.0	5.00	10.00	6.10	0.00	6.20	0.00
10.0	0.10	4.90	5.80	0.20	4.55	+0.05
10.0	0.25	4.75	5.70	0.30	4.38	0.12
10.0	0.50	4.50	5.50	0.50	4.25	0.25
10.0	1.00	4.00	5.25	0.75	4.00	0.50
10.0	2.00	3.00	5.00	1.00	3.80	0.70
10.0	3.00	2.00	4.65	1.35	3.75	0.75
10.0	4.00	1.00	4.25	1.75	3.72	0.78
10.0	5.00	0.00	4.15	1.85	3.72	0.78

TABLE 6. THE INFLUENCE OF AN ACID DYE UPON THE PH OF *Escherichia coli*

ml. of H-bacteria	ml. of Stain	ml. of Water	pH of H-bacterial Suspension treated with Phloxine	
			Actual pH	Increase in pH
5.0	0.00	2.00	4.90	0.00
5.0	0.10	1.90	6.08	0.78
5.0	0.25	1.75	6.20	0.90
5.0	0.50	1.50	6.23	0.93
5.0	1.00	1.00	6.15	0.85
5.0	2.00	0.00	6.00	0.70
0.0	5.00	2.00	5.30	0.00

THE INFLUENCE OF AN ACID STAIN ON THE PH-VALUE OF ESCHERICHIA COLI

Since acid stains act as anions (Conn, 1940) they should have the opposite effect on the pH-value of a suspension of H-bacteria to that of basic stains. The influence of phloxine upon the pH-value of suspended *Escherichia coli* cells is shown in Table 6. It is evident that the pH-value increased as the quantity of phloxine increased up to a certain maximum and that further additions of the stain did not increase the pH-value. When 1 ml. or more of phloxine was added to the bacterial suspension the pH-value decreased. This may be

attributed to having an excess of the stain which has a pH-value below that of the stain-bacteria mixture.

Discussion

The data collected in this investigation agree with the views set forth in the introduction; namely, that when a stain combines with a bacterial cell it replaces some ion already adsorbed by the cell. This was demonstrated by using bacteria saturated with adsorbed Mg^{++} or H^{+} at specific concentrations. When a basic stain was added the adsorbed Mg^{++} or H^{+} was released and found in the supernatant liquid, while the stain became adsorbed by the cell. This replacement indicates that a stain is adsorbed at the same position as the inorganic cations. Assuming that stains have a similar mechanism of attachment to a bacterial cell as inorganic cations, this should establish a common basis for evaluating the effect of the two types of substances on bacteria.

Conclusions

The data obtained in this investigation support these conclusions:

1. The reaction of stains with bacteria is an adsorption exchange process, reaching stoichiometrical proportions. Two methods were used to show that staining is an exchange reaction.

2. Basic stains act as cations, replacing similarly charged ions from the bacterial system.

3. Basic stains decrease while acid stains increase the pH value of a suspension of H-bacteria (i.e. bacteria treated with H^{+}).

4. From the increase in acidity obtained by displacing H^{+} with a basic stain there appears to be a maximum exchange value.

5. Stains appear to react with the bacterial cell at the same positions as do inorganic cations.

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P-AMINODIMETHYLANILINE MONOHYDROCHLORIDE AS AN INDICATOR OF MICROBIAL ACTION ON FATS

C. H. CASTELL, *Department of Bacteriology, Ontario Agricultural College, Guelph, Canada*

ABSTRACT.—**Flooding p-aminodimethylaniline monohydrochloride on fat emulsion agar inoculated with certain types of microorganisms frequently results in marked color changes in the fat globules. It is shown in this paper that the colors result from the increased solubility in fat and fatty acids of this dye as it becomes oxidized. Some of the acids oxidize the dye on contact and therefore color very quickly; fats become colored only when some other agent oxidizes the dye. The characteristic color reactions with certain fats and fatty acids are described for various degrees of oxidation of the dye; this suggests the explanation for the colors observed in the inoculated globules flooded with this dye. A table is included showing the colors in globules of oil that were inoculated with 39 pure cultures of bacteria.**

For detection of fat splitting bacteria, Jensen and Grettie (1937) have combined the oxidase test described by Gordon and McLeod (1928) with an unstained oil emulsion agar. With this medium they are able to identify lipolytic organisms by the transparent or translucent zone surrounding the colonies. Those organisms which incite or aid in the production of oxidative rancidity are recognized by their colony color reaction when flooded with dyes of the paraphenylenediamine series.

Castell and Bryant (1939) have observed that at least one of these oxidase detecting dyes, *p*-aminodimethylaniline monohydrochloride, also produces a variety of color changes in fat globules undergoing decomposition. These can be observed under the low power lens of the microscope, and they vary from red, yellow, brown or black to colorless. By examining the action of this dye on triglycerides, fatty acids and other organic compounds, it was shown that the reduced dye solution had little effect on the fats but was readily absorbed and oxidized by the fatty acids, producing shades of color of light brown to black. The color darkened with each decrease in the molecular weights of the acids. It was concluded that the presence of free acid in the decomposing fat globules enabled them to absorb the dye from the surrounding agar medium. Further work, including attempts to discover the reason for green, blue and other color

changes in fat globules inoculated with certain types of bacteria and stained with this indicator, is summarized in this article.

Color Reactions of the Dye. When a fresh 0.5% aqueous solution of this dye is prepared it is almost colorless. On standing, it gradually becomes oxidized to shades of pink rose, dark red and reddish black. It becomes a dark purple to bluish black on further exposure. Up to the point where the solution becomes dark red, it can be reduced again. Once the blue black stage has been reached, however, the color change does not appear to be reversible.

Triolein and olive oil will not absorb the dye in its reduced form. However, as it becomes oxidized to shades of dark red it can be readily absorbed by the fat from the aqueous solution, coloring the oil a deep red. Neither triolein nor olive oil is colored by the dye when it has been oxidized to the non-reversible bluish-black stage.

Oleic acid is colored a light brown or amber color when mixed with the dye in a pale pink form. As the dye is further oxidized until the dark red or purple stage is reached, the depth of brown color increases as oleic acid is added. Mixed with the dye in its dark blue stage this acid remains colorless.

The insoluble saturated fatty acids turn to shades of greyish brown and black when mixed with the pale pink dye solution. The fewer the number of carbon atoms in the acids, the more intense the color reaction. When added to dye solutions oxidized thru shades of dark red, purple and blue, the acids also take on purple and blue shades.

Significance of Color Reactions in Inoculated Fat Globules. The application of these results to the color changes in the fat globules of an inoculated oil emulsion agar suggests the following interpretations:

(1) Red globules under, or surrounding a colony, indicate that the bacterial oxidase has oxidized the dye in the immediate vicinity of the colony, to the stage where it has become fat soluble. When the plate is left exposed to the air after the dye has been added, all the globules will turn red in a few hours. There is no reason to believe that this color change results from any change in the fat. However, it is not unlikely that enzymes actively oxidizing the dye may also aid in the oxidation of unsaturated fats.

(2) The presence of amber or buff colored globules suggest fatty acids, more especially oleic or some similar acid.

(3) Dark brown or black globules unaccompanied by an outer ring of red globules would suggest the presence of caproic, capryllic or capric acids and indicate an oxidase negative colony.

(4) Blue and green globules show the presence of these same saturated acids together with a strongly oxidase positive bacterial colony.

(5) When globules around or under the colonies become colored at the same rate as globules in uninoculated portions of the plate, the bacterial colony neither hydrolizes the fat nor is oxidase positive.

(6) When the globules remain colorless around a colony long after those in other portions of the plate have become red, the bacterial colony is exerting a strong reducing action on the surrounding medium.

Color Reactions With Pure Cultures of Bacteria. Fat emulsion medium was prepared by shaking olive oil in standard nutrient agar. This was poured into sterile petri plates and when cool, streaked with pure cultures of bacteria. After incubation at 25°C. (77°F.) for four days, the plates were flooded with a freshly prepared 0.5% aqueous solution of *p*-aminodimethylaniline monohydrochloride. After standing for two or three minutes the dye was poured off and the plates were examined microscipocally after one-half, one, two and six hours.

Table 1 shows the results of these observations compared with the color reactions of Nile blue sulphate in the same medium with the same organisms.

It will be noted that the results of this dye on the various pure cultures used, agree fairly accurately with those obtained by using Nile blue sulphate as the indicator. With some organisms the globules were colored differently in different areas. The globules surrounding the colonies of the *Pseudomonas* group and *Phytomonas campestris*, were colored a dark red; those under the colony were shades of blue and buff.

Similar plates were examined after 24 days' incubation. Several cultures, especially *Escherichia coli*, *Aerobacter aerogenes* and *A. cloaceae*, which had formerly given negative reactions, turned dark brown when flooded with the dye solution, indicating acid production.

For the time being this dye is not recommended as a substitute for Nile blue sulphate or copper sulphate as an indicator for lipolytic bacteria. However, the dye has been shown by Castell and Garrard (1939) to have an increasingly important place in certain phases of dairy bacteriology and has been already used in conjunction with fat emulsion media by Jensen and Grettie (1937). It also appears to have a value in yielding information not given by ordinary dyes used for detecting lipolysis.

TABLE 1. COLOR REACTIONS IN FAT GLOBULES INOCULATED WITH PURE CULTURES OF BACTERIA AND FLOODED WITH *p*-AMINODIMETHYLANILINE MONOHYDROCHLORIDE AND COMPARED WITH THE COLOR REACTIONS OF THE SAME ORGANISMS WITH NILE BLUE SULPHATE AS AN INDICATOR

Organism	Red	Blue	Amber colored	Blue with Nile blue sulphate
Bacillus subtilis	–	–	–	–
Bacillus mycoides	–	+?	–	+?
Bacillus panis	–	–	–	–
Bacillus cereus	–	+	–	+
Bacillus graveolens	+	–	–	–
Bacillus mesentericus	++	–	–	–
Staphylococcus aureus	–	–	++	+
Staphylococcus citreus	–	–	+	–
Streptococcus lactis	–	–	–	–
Micrococcus conglomeratus	+	–	–	–
Diplococcus capsulatus	–	–	–	–
Sarcina lutea	–	–	+?	–
Sarcina aurantiaca	–	–	–	–
Erwinia carotovora	++	–	–	–
Rhizobium radicicola	++	–	–	–
Phytomonas campestris	+	+	–	+
Achromobacter putrefaciens	++	–	–	–
Achromabacter lipolyticum	+	+	+	+
Pseudomonas fluorescens	++	+	+	+
Pseudomonas aeruginosa	++	+	–	+
Pseudomonas fragi	++	+	–	+
Pseudomonas mucidolens	++	+	–	+
Alcaligenes viscosus	–	–	+	–
Alcaligenes lipolyticum	+	+	–	+
Alcaligenes faecalis	++	–	–	–
Brucella abortus	++	–	–	–
Eberthella typhosa	–	–	–	–
Aerobacter aerogenes	–	–	–	–
Aerobacter cloaceae	–	–	–	–
Salmonella (9 species)	–	–	–	–
Proteus vulgaris	–	–	–	–
Escherichia coli	–	–	–	–

++strong reaction
+weak reaction
– negative reaction

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GORDON, J., and MCLEOD, J. W. 1928. The practical application of direct oxidase reaction in bacteriology. J. Path. and Bact., **31**, 185.

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NOTES ON TECHNIC

A Note on the Use of Synthetic Glycerol (Shell) in the Clearing of Embryos in Toto.—Recently Williams[1] announced the synthesis of glycerol from petroleum gases which has proven of special interest both commercially and pharmacologically. Williams stressed that the synthetic glycerol (Shell), tested pharmacologically proved to produce no ill effects, despite the fact that allyl chloride and alcohol, both highly toxic materials, were intermediate products in the synthesis. Hart[2] has substantiated these findings in a pharmacological comparison between synthetic glycerol and glycerol produced from natural oils.

Since natural glycerol is used extensively in certain of the well-known clearing processes on embryos *in toto*, it was of interest to the author to compare the synthetic glycerol (Shell) with that of natural glycerol. The procedure was essentially the same as the well-known Schultz modification of Spalteholtz' technic, with the exception of the staining procedure described below.

Initial clearing of the embryos was done with 5% KOH (with smaller specimens only 1–2% KOH was used to prevent disintegration of the tissues) and required 1 to 2 days, depending upon the size of the specimens. Staining of ossification centers was accomplished with a 0.1% alizarin red S solution (National Aniline) in distilled water. The alizarin red was added, drop by drop, with constant stirring, to a 2% Na_2CO_3 solution, until a uniform reddish-purple mixture was obtained. The specimens were transferred directly from the KOH to the staining mixture. Complete staining usually required from 12 to 24 hours in the above staining medium. This method of staining has proven very satisfactory in all specimens stained for bone preparation, with very little or no fading of the dye, even after several months of permanent mounting in glycerin.

After completion of the staining, the specimen is transferred to a 25% solution of synthetic glycerol (Shell) and allowed to remain in the glycerol from 3 to 5 days (again depending upon the size of the specimen). The author has used embryos of rat, pig and calf, and has found the entire skeletal system plainly visible after a few days immersion in the 25% synthetic glycerol. The specimen is then carried over to a 50% synthetic glycerol for 3–7 days. Final clearing

[1]Williams, E. C. 1938. Modern petroleum research. Ind. Eng. Chem., **16**, 630.
[2]Hart, E. Ross. 1939. A pharmacological comparison between synthetic glycerol and glycerol prepared from natural oils. Univ. of Calif. Publ. in Pharmacology, **1**, 271–4.

is done in pure synthetic glycerol; the specimen mounted for permanent preservation by the method previously described.[3] Precaution is taken to add a small amount of crystalline thymol to all glycerol solutions to prevent contamination by fungi.

Comparison of synthetic glycerol (Shell) with that of natural glycerol in the clearing of embryos *in toto* has proved that the synthetic product removes excess dye from the soft tissues, particularly in 25% synthetic glycerol. Repeated observation with natural glycerol fails to reveal any appreciable removal of excess dye in comparison with the synthetic glycerol, unless alcohol is added to the natural glycerol. Synthetic glycerol (Shell) is equally desirable, if not somewhat superior, in the clearing of embryos *in toto*.

The author wishes to express his sincere appreciation to the Shell Development Company of Emeryville, California, for the generous supply of synthetic glycerol used in this investigation.—CLARENCE W. NICHOLS, JR., Santa Cruz, Calif.

A METHOD FOR MAKING PERMANENT MOUNTS OF PORTIONS OF DECOLORIZED WHOLE LEAVES.[4]—A wide-mouthed bottle is a suitable container to carry out the processes of decolorizing, dehydrating and clearing. The leaves are decolorized by a method previously described by the writer.[5]

After decolorizing, the leaves are dehydrated with ethyl alcohol in the usual manner, and the dehydration completed in carbol-xylol which is made by adding one part phenol crystals to three parts xylol. The leaves are then cleared by treating with two changes of pure xylol.

After clearing, the leaves are removed from the bottle one at a time and placed in a shallow dish of xylene. Small portions of the leaf from 5–10 mm. square are cut out with scissors, mounted on a clean slide in a drop of hyrax and covered with a cover glass. When these preparations are examined under the microscope, the different layers of leaf tissue may be observed by simply raising and lowering the objective.—JAMES C. BATES, Kansas State College, Manhattan, Kansas.

[3]Nichols, C. W., Jr. 1940. A simple method for mounting embryological material. Stain Techn., **15**, 119.

[4]Contribution No. 408, from the Department of Botany and Plant Pathology, Kansas State College of Agriculture and Applied Science.

[5]Bates, J. C. 1931. A method for clearing leaves. Amer. Nat., **65**, 288. (See p. 44 of this issue.)

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

MICROSCOPE AND OTHER APPARATUS

SEIFRIED, O. **Neuzeitliche Sammlung und Ordnung histologischer Schnitte.** *Zts. wiss. Mikr.*, **56**, 367–71. 1939.

A new filing system for microscopical preparations is described. It consists of card board units (length 14½ in., width 4 in., metal bound) that accomodate 10 slides. The lower two-thirds of the slides fit into paper pockets which afford protection to the preparations. A special cabinet is provided having a series of drawers 14½ × 18 in.—*J. M. Thuringer.*

PHOTOMICROGRAPHY

ABRAHAMSON, E. M. **An inexpensive microphotographic camera.** *Science*, **91**, 510. 1940.

A "Univex" camera, model AF (price $1.00) can be adapted for photomicregraphical uses as follows: Set the shutter for "time" and open it. With nail and hammer strike a smart blow at the lens and shake out the chips. Draw out the wire frame used as a finder, and remove the horizontal section by bending at the angles till breakage occurs. Bend the two side pieces down along the front of the lens board, and curve slightly to receive the ocular of the microscope. Remove the back of the camera, connect to the microscope tube, and focus by means of a ground glass or tracing paper 33 × 45 mm. Introduce a role of film (price $0.10) and take six exposures at varying times. At least one exposure will give a sharp negative; no rules can be given since the time of exposure depends on the illumination.—*J. A. de Tomasi.*

HELLWIG, C. ALEXANDER. **Routine microfilming of tumor slides.** *Amer. J. Clin. Path., Tech. Suppl.*, **4**, 95–9. 1940.

The following method is proposed for routine microfilming: A 12.5 cm. metal focusing tube is fitted to the eyepiece of the microscope by an adapter ring. A ground glass at the top allows centering of the light source and focusing the field. The camera, which is also 12.5 cm. long, consists of a Bantam back attached to a Bakelite bellows and is held on the eyepiece of the microscope by gravity. The lower end of the camera, made of heavy brass, is provided with a dark slide. The best light source is a 100 watt Spencer microscope lamp with an iris diaphragm and a holder for Wratten filters B and G. It is focused on the plane mirror 8″ away. Eastman Panatomic film F 828, 28 × 42 mm., is used. The following procedure is recommended: Set the focusing tube on the eyepiece. Focus the margin of the iris diaphragm sharply on the center of the ground glass. Switch off the light. Replace the tube by the camera. Remove the dark slide and expose the film by switching the light on and off. Replace the dark slide and remove the film. Develop preferably with Champlin No. 16 developer at 63° F. for 9 min. Fix 15 min. and wash in running water. Remove droplets of water by a piece of wet cotton, and dry by a fan. Use a fixed focus enlarger to produce 2½ × 3″ pictures on Kodabrom No. 4 paper or lantern slides. Develop in Eastman D–72.—*G. H. Chapman.*

McCLENDON, J. F. **Inexpensive microphotographic records.** *Science*, **92**, 134. 1940.

The suggestion made (Abrahamson, *Science*, **91**, 510, 1940) that the lens of a "Univex" camera be destroyed in order to obtain a focus in photomicrographical work is opposed by the author. If the camera is placed in the same position

as the eye after focusing the microscope, the camera will be in focus. To center it in position, a paper or tin tube may be applied to the front of the camera, and slid down over the eyepiece. The tube is secured with liquid solder, or any other quick-drying nitrocellulose adhesive. The time exposure is usually about 1 sec.—*J. A. de Tomasi.*

MICROTECHNIC IN GENERAL

HAMILTON, J. M. **Isobutyl methacrylate as a mounting medium for histological preparations.** *Science,* **92,** 44. 1940.

Celloidin sections, 30 × 50 mm. and 25 μ thick, of brain tissue mount unsatisfactorily in isobutyl methacrylate polymer (Du Pont Co.). This material in xylene solutions of various concentrations, when used without a cover glass, causes the sections to warp on hardening, and the resulting surface is rough. Under a cover glass, the sections crack and split. The thionin stain for Nissl cells remains unaffected, but the blue of the myelin sheaths stained with the Weil method turns a dull gray and some of the details are lost.—*J. A. de Tomasi.*

HOFFSTADT, RACHEL, and OMUNDSON, DOROTHY. **A convenient apparatus for the manipulation of eggs in the study of the chorio-allantoic membrane.** *Science,* **91,** 459–60. 1940.

A search has been made for a convenient arrangement usable in high power microscopy for studying the virus of infectious myxomatosis and its action on the chorio-allantoic membranes of chick and duck embryos. As a result a simple apparatus is described which is composed of appliances available in any laboratory. After culturing the virus on the membrane, an opening of 2–3 cm. in diameter is made in the egg shell, and a wall of some 2 mm. is built around it with a mixture of equal parts of vaseline and paraffin. A few drops of saline are placed on the membrane, and a cover slip is sealed to the paraffin. Another small opening in the shell allows tight application by paraffin of a glass tube in correspondence with the egg air sac. By means of rubber tubing and a clamp, air can be blown gently into the air sac, and the level of the membrane raised or lowered according to need.—*J. A. de Tomasi.*

JENSEN, H. W. **A speedier and less costly method of concentration in nitrocellulose imbedding.** *Science,* **91,** 509–10. 1940.

Any laboratory using celloidin as an imbedding agent will soon accumulate amounts of it that can again be put to use in the following way: Redissolve in ether-alcohol and let settle for a few days. Decant into petri dishes to a depth of ¼ in. Let it solidify and cut into 1 in. squares. Gather pieces on a No. 1 pin, and dry completely. These pins may be used instead of the usual troughs attached to the inside of the cork in the imbedding bottle. Advantages: reduced cork leakage; concentrating of the 10% celloidin shortened by several days; for most cytological materials, celloidin stages cut down to 2, 6, 10% steps without apparent damage.—*J. A. de Tomasi.*

SUNTZEFF, V. and SMITH, IRENE. **The use of plastic as a substitute for cover glasses.** *Science,* **92,** 17–8. 1940.

The shortage of cover glasses caused by the conflict in Europe has again suggested the use of plastic materials in their stead. The authors suggest as a suitable plastic "Vue Pak" (Monsanto Chemical Co.) which can be obtained in sheets 0.005 in. or 0.127 mm. thick, comparable to the thickness of No. 1 cover glasses. The refractive index is 1.49–1.50, very close to that of glass, and the resistance to heat is 140–180° F. Also, the effect of sunlight, weak acids and alkalies is negligible. Dried slides thus covered remain unchanged when repeatedly held at 110–115° F. for 12 hr., and cooled. The plastic sheet is cut to cover glass size, and kept between sheets of tissue paper; for cleaning, it may be dipped in 50% alcohol and dried immediately. As the slips have a tendency to curl, the drying should be carried out slowly at room temperature. By substituting isobutyl methacrylate polymer (Du Pont Co.) for Canada balsam as a mounting medium, drying does not require more than a day, and results in the whole are improved.—*J. A. de Tomasi.*

DYES AND THEIR BIOLOGICAL USES

CHALKLEY, L. **Organic mercury derivatives of basic triphenylmethane dyes.** *Science*, **91**, 300. 1940.

Owing to the characteristics of the basic triphenylmethane dyes, development of organic mercury derivatives seems desirable and promising in such fields as bacteriology and pharmacology. It is found that derivatives can best be prepared by a two-stage process where a dye intermediate is first mercurated, and the resulting compound then converted into the dye. A small amount of dicyanomercury malachite green has thus been prepared, and is being offered freely to interested experimenters.—*J. A. de Tomasi.*

FORSTER, R. PH. **A renal clearance analysis of phenol red elimination in the frog.** *J. Cellular and Comp. Physiol.*, **16**, 113–22. 1940.

Inulin clearance, a measure of glomerular filtration, was compared with simultaneous phenol red clearance to analyse the tubular secretion of the dye.

For phenol red concentrations in the plasma below 0.5 mg. per 100 ml., 97% of the total dye was eliminated by the kidney tubules. The maximal rate of tubular secretion, 5.0 mg. per kilogram body weight per hr., was attained at a plasma concentration of approximately 5 mg. free phenol red per 100 ml. The phenol red to inulin clearance ratio averaged 7.0 at a total phenol red concentration in the plasma of 1.0 mg. per 100 ml., and below 1.0 at a plasma concentration of 40 mg. phenol red per 100 ml.—*L. Farber.*

FOX, C. L. **Is sulfanilamide bacteriostatic under anaerobic conditions?** *Science*, **91**, 477–8. 1940.

Methylene blue, introduced either initially or after full growth of *Escherichia coli*, proves that anaerobic, reducing conditions interfere with sulfanilamide bacteriostasis. The medium in which the organism is grown is that suggested by Quastel, Stephenson, and Whetham (*Bioch. J.*, **19**, 304, 1925). In the simple lactate medium, where reducing conditions prevail, sulfanilamide has no effect upon growth; in the "anaerobic" lactate-nitrate medium in which the methylene blue is not reduced, the bacteriostatic action of sulfanilamide is evident. These findings may help to interpret the unsatisfactory effect of sulfanilamide therapy in mastoiditis and other closed infections which are thought to be accompanied by anaerobic glycolysis.—*J. A. de Tomasi.*

HÖBER, R., and BRISCOE-WOOLLEY, P. M. **Further studies on conditions determining the selective renal secretion of dyestuffs.** *J. Cellular and Comp. Physiol.*, **16**, 63–70. 1940.

Sulfonic acid azo-dyes having a bilateral elongated molecule with the sulfonic acid groups on one half of the molecule are secreted by the proximal tubules of the excised and Ringer- or blood-perfused frog kidney. Triphenylmethane dyes are not secreted by the kidney perfused with Ringer solution, but some are secreted by the freshly excised kidney. Furthermore, perfusion with Ringer solution for 1 hour prior to excision inhibits or greatly diminishes the secretion of those triphenylmethane dyes previously secreted, but does not affect the secretion of the sulfonic acid azo-dyes. The authors conclude that two mechanisms are involved in the secretion of dyes, one depending on a physico-chemical affinity between dye and cell, the other involving some component in the cell environment which is not present in Ringer solution.—*L. Farber.*

ANIMAL MICROTECHNIC

CRAIG, JANE S. **A simple stain for tissue cultures.** *Science*, **92**, 226. 1940.

In hanging drop tissue cultures in plasma, most stains suitable for the cytoplasm also stain the plasma of the clot. The following method, for brain tissue, reveals clearly the finest processes of the cells, nuclei, and certain cytoplasmic inclusions: Remove from the cover slip all paraffin and vaseline with cotton pledgets soaked in chloroform. Fix 24 hr. in 10% neutral formalin, or absolute alcohol. Stain 1 hr. in 1% aqueous toluidine blue, and wash in 2 changes of distilled water. Dehydrate 2–3 min. in 85%, 2–3 min. in 95% alcohol. Differentiate 5–10 min. in absolute alcohol under the microscope. Clear in xylene, and mount in balsam, or nevillite.—*J. A. de Tomasi.*

EINARSON, L., and BENTSON, K. **Bermerkungen zur progressiv-selektiven färberischen Darstellung der Nervenzellen in Paraffin- und Zelloidinschnitten.** *Zts. wiss. Mikr.*, **56**, 265–72. 1939.

The authors emphasize the wide range of usefulness of the Gallocyanin stain for nervous tissues. Prepared according to the original formula, it stains only the Nissl substance, nuclear chromatin, nucleoli, and nuclei of glia cells.

Celloidin sections are stained by the following procedure: Dissolve 5 g. aluminum sulfate and 5 g. chromalum in 100 ml. of dist. water. Add 0.5 g. anthracene blue. Shake and bring to a boil for 10–15 min. (The reaction of this solution is about pH 1.95; but it is adjusted by means of buffers, as explained below.) Stain for 24–48 hr. Paraffin sections are stained for 48 hr. No differentiation is required and over-staining does not occur.

Lowering the pH from 2.09 to 1.20 produces selectivity which gradually eliminates every trace of co-staining any other but the specific substances. Conversely, raising the pH from 2.09 to 4.46 increases co-staining of glia cells and fibrillar elements, cytoplasm, and cell processes. At pH 2.60–3.80 histological pictures of exceptionally high quality are produced.

The buffering of the stain is given in a special table. For human cerebral-cortex stain buffered at pH 1.95–2.59 is used; for mesencephalon, pons, medulla oblongata, and cerebellum, pH 2.68–2.73 and for spinal cord pH 2.73–3.25. After staining, section is washed in distilled water, finished thru alcohol, carbol-xylol, and balsam. This technic is based on formalin-fixed material, and is suitable both for paraffin and celloidin sections. Zenker-fixed material yields especially good cytological pictures; however, the unspecific co-staining is considerably modified.—*J. M. Thuringer.*

FOGG, L. C., and WARREN, S. **The centriole in radiated tumor tissue.** *Science*, **91**, 528–9. 1940.

Previous work on the cytology of Walker rat carcinoma 256 had not pointed out the presence of centrioles. X-ray irradiation of such material reveals paired bodies in the interkinetic stage of the cells of this tumor. The type and dosage of radiation is as follows: 200,000 volt X-ray; 50 cm. distance; filter 0.5 mm. Cu, 1 mm. Al; one dose 2400 r.; rate of irradiation, 40.6 r. per min. After 18, 48, 72, 96, and 120 hr. of irradiation, the animals are killed and the tissue fixed either in Bouin or Zenker. Staining is most satisfactory with: Heidenhain's iron hematoxylin and eosin, eosin Y and methylene blue, or phosphotungstic acid hematoxylin. A quantitative analysis of cells showing centrioles reveals that irradiation at various intervals detects a wide variation in the frequency of multiple centrioles. Their number increases up to 72 hr., then sharply decreases to a normal level after 120 hr. of treatment.—*J. A. de Tomasi.*

FOLEY, JAMES O. **A new silver method for staining nerve fibers in blocks of nervous tissue.** *Anat. Record*, **73**, 465–72. 1939.

A silver method for staining peripheral nerves, designed to display the smallest axones distinct and separate, is described as follows: Fix the stretched nerve 24 hr. at 4° C. in the following solution: potassium bichromate (3–5% aqueous solution), 49 cc.; ammonium hydroxide (28%), 1 cc.; pyridine (concentrated), 15 cc.; ethyl alcohol (95%), 50 cc. Wash 15–30 min. in each of a series of alcohol-pyridine mixtures, composed of 15 vol. pyridine to 85 vol. dilute alcohol, the alcohol in the first bath being 50%, in the 2nd 40%, the 3rd 30%, the 4th 20% and the last 10%. (In the last solution no yellow color should be extracted from the tissue.) Pass thru an ascending series of aqueous pyridine (10%, 20%, 30%, 40%, 50%), 15–30 min. each. Remove tissue from stretcher, snip off knot at one end, and thread thru strips of cerebral cortex preserved in 50% pyridine. Pass thru an ascending series of aqueous pyridine (60%, 70%, 80%, 90%), at least 30 min. each; absolute pyridine 24 hr., 2 or 3 changes. Trim with razor 3–5 mm. in diameter. Run thru a descending aqueous pyridine series (from 90% to 10% in steps of 10%), 15–30 min. each. Wash 36 hr. in distilled water, changing every half hour during the working day. Leave 3–5 days in $AgNO_3$ at 37.5° C. Rinse few seconds in water. Treat 48 hr. with Ranson reducer (4% pyrogallic acid in 5% formalin). Dehydrate in alcohols and imbed.

The author recommends his celloidin paraffin embedding for thin sections and cytological details. For 10–20 μ sections he recommends imbedding in butyl alcohol and paraffin. Some sections should be toned and counterstained under the microscope.

A method quite similar to the above is given for central nervous system tissues. Such tissues are perfused 1–2 hr. with the fixing agent and are then placed in fresh fixative in the ice box for 48 hr. The silvering is increased to 5–7 days. Embedding in butyl alcohol and paraffin is recommended. The author states that celloidin or dioxan will tend to extract the silver. Sections may be toned and counterstained for various studies.—*S. I. Kornhauser.*

GRAFFLIN, ALLAN L. **Histology of the thyroid and parathyroid glands in the mountain gorilla, with observations upon autofluorescence, fat and pigment.** *J. Morphol. & Physiol.*, **67**, 455–70. 1940.

Tissues were fixed in 5% formalin, in which they remained for about 7 months before this study was made. Frozen sections were mounted unstained in C.P. glycerin and studied under both the ordinary and the fluorescent microscopes. Sections for the study of fat were stained either for 5 min. with a saturated solution of Sudan III in 70% alcohol containing 1% KOH, or for 24 hr. in a saturated solution of the dye in 40% alcohol. Routine histological preparations were made by embedding tissues in paraffin, via absolute alcohol and chloroform, serially sectioned at from 5 to 10 μ, and stained with hematoxylin and eosin.—*Elbert C. Cole.*

HESS, M. **The localization of acid azo dyes in tumours.** *J. Path. & Bact.*, **51**, 309–11. 1940.

Necrotic changes in two transplantable carcinomas of mice and rats were clearly demonstrated after the intravenous injection of Chicago blue 6B (source not stated). For mice, 15 mg. of dye in 0.5 cc. of distilled water served as the dose, and for rats, 60 mg. in 1.5 cc. This was followed by fixation in Susa for 2 hr., dehydration in alcohol not longer than 5 hr., and 2 changes of chloroform. Paraffin sections were treated with iodine and examined both unstained and after light staining with carmalum.

After 24 hr., necropsy revealed diffuse blue in most organs and tissues, but brain, lungs and spleen appeared dye-free. Carcinoma cells were unstained, but fibroblasts and stroma cells in the tumor were laden. Degenerating cells were diffusely stained. Mesodermal elements thruout the body stored large amounts of dye.—*S. H. Hutner.*

KRAJIAN, ARAM A. **Frozen section method for preparation of permanent tissue sections.** *Amer. J. Clin. Path., Tech. Suppl.*, **4**, 82–8. 1940.

Frozen sections may be made in place of paraffin or celloidin sections if the following suggestions are followed: The microtome knife should have a shorter bevel on the under side. In cutting sections, skin, capsule or other covering must face the knife. Put a drop of water on the microtome. Press the block firmly on it and freeze slowly and evenly until about one-half of the block is frozen. Cut off the unfrozen portion and shave. (If the tissue is too cold, it will be hard and white. If too soft, it will disintegrate in water. If it is cut too fast, the sections will curl or break into shreds. Firmer tissues give thinner sections for the same setting of the microtome.) Soak loose texture tissues in a Stender dish containing fresh serum. Cover any pieces that float with cotton. Put the dish on top of the paraffin oven overnight. Without washing cover the block with dioxan 3–5 hr. For fatty tissues cut sections 15 μ, leave them on the edge of the knife, apply 2–3 drops of 95% ethanol 1 min., and transfer them to water by the tip of the little finger. If this does not make the sections spread, keep them in the ethanol 30–45 min. and then in water. If this does not help, put the block in equal parts of acetone and 95% ethanol, fix again in formalin and then cut. After affixing sections to the slide, drain 15–60 sec. but do not let them become dry. Hold the slide at a slight angle, add 2 drops of anhydrous isopropanol (Union Carbide and Carbon Company) or absolute ethanol. Blow until the section is white and then blot with Whatman filter paper No. 2. Do not blot mucoid tissues. Flood the section 1–10 min.

with isopropanol. Drain carefully, blot, and dip rapidly twice in the following solution: equal parts absolute ethanol and ether, 83 ml.; thick celloidin, 7 ml.; gum mastic, 1 ml. (gum mastic, 25 g.; 95% or absolute ethanol, 35 ml.; stand 1 week). Blow gently. Stain immediately in alum hematoxylin, 3–5 min. Rinse in tap water until blue (1–2 min.). Destain in the following solution: HCl, 1 ml.; 70% ethanol, 100 ml. until no more color runs. Rinse in tap water. Dip once or twice in 2% ammonia water until blue. Dip in tap water. Wipe the slide but not the tissue. Put the slide in 95% ethanol or 88% isopropanol 1–10 min. Add 2–3 drops of anhydrous ethanol or isopropanol. Repeat this addition twice. Dip several times in eosin until the background is stained evenly red (10–30 sec.).

The eosin stain is prepared as follows: aq. eosin, 5 g.; dist. water, 10 ml.; glacial acetic acid, 10 ml.; conc. HCl, 2 ml. Mix with a glass rod. Put the mixture in a 56° C. oven 12–16 hr. Dissolve the mass in 10 ml. absolute ethanol or anhydrous isopropanol and 20 ml. acetone. Stir. Keep 5 hr. at 56° C. Let settle. Remove the clear supernatant and add it to 1,500 ml. of the following: phenol crystals, 1 part; neutral xylene, 3 parts. Use the clear portion of this eosin solution. (For standardization see *Arch. Path.*, **25**, 376–7, 1938.)

Put the stained slide in carbol-xylol 3–4 min. and drain completely. Put in 3 changes of xylol, 2 min. each, draining each time. Mount in neutral gum dammar (Eastman Kodak company, saturated solution in neutral xylol). If sections should become detached from the slide during the staining process, flood the section on the slide with isopropanol 2–5 min., drain, blot, and dip twice in the celloidin solution.—*G. H. Chapman.*

MORRISON, MAURICE, and SAMWICK, A. A. **Restoration of overstained Wright films and a new method of staining blood smears.** *Amer. J. Clin. Path., Tech. Suppl.*, **4**, 92–3. 1940.

Poorly stained blood films may be corrected by flooding with Wright's stain for 15–30 sec. and rinsing with distilled water. Fresh films may be more easily stained by the following technic: Cover with Wright's stain and allow to evaporate. Add more Wright's stain, 15–30 sec. Wash with distilled water.—*G. H. Chapman.*

SILLS, BERNARD. **A simple aid in making a blood smear.** *J. Lab. and Clin. Med.*, **25**, 1302. 1940.

The following device is described which overcomes many of the difficulties of the Ehrlich two-cover glass method: Place two perfectly clean cover glasses face to face. Gently warm the tip of a waxed stick (the Mexican wax match is satisfactory), place it in contact with the edge of the top cover glass and allow it to harden. Lift the top cover glass with the stick as a handle, touch it to the drop of blood secured in the usual manner, and place it on the lower cover slip so that none of the corners coincide. As soon as the drop spreads, pull the cover glasses apart by grasping the lower one by the corners and the upper one by the stick. The glasses need not be touched with the fingers until after the drop is spread.—*John T. Myers.*

PLANT MICROTECHNIC

BATES, JAMES C. **A method for clearing leaves.** *Amer. Nat.*, **65**, 288. 1931.

The following method has proved quite effective for clearing and decolorizing whole leaves darkened by preservatives and containing considerable quantities of tannin.

The leaves to be cleared are placed in a test tube and covered with a saturated solution of chloral hydrate for 48 hr. or longer. The chloral hydrate is then poured from the material and enough $KClO_3$ added to fill the bottom of the test tube to about ½ in. The material is then covered with concentrated HNO_3 and let stand until the leaves begin to change color. From 10–30 min. is usually sufficient. The HNO_3 is then poured out, leaving the $KClO_3$ and leaves in the test tube, and the material again covered with a saturated solution of chloral hydrate. In this solution there will be a continuous liberation of chlorine gas and within less than a week the leaves should be quite transparent. If necessary the HNO_3 treatment may be repeated.—*Reprinted from original.*

CHRISTENSON, B. V., and STOKES, R. C. **A pharmacognostical study of Serenoa serrulata (saw palmetto).** *J. Amer. Pharm. Assoc.*, 29, 199–206. 1940.

On account of the presence of extremely thick cell walls and numerous lignified stone cells in the fruit of the saw palmetto, it was necessary to devise special methods of treatment for rendering it suitable for sectioning. The seed was removed after making a transverse incision around the middle portion of the fruit and lifting off one-half. The hard seed was boiled in water for 24 hr., then placed in a 25% solution of HF for a week, thoroly washed in water and left for a week longer in equal parts of 95% alcohol and glycerin. The pericarp was steamed on a water bath for 2 hr. and cut into longitudinal sections. Paraffin sections 15 μ thick were satisfactory for histological study.—*Merritt N. Pope.*

McLEAN, ROBERT S., and IRELAND, EDWARD J. **Rapid staining methods in plant histology.** *J. Amer. Pharm. Assoc.*, 29, 318–21. 1940.

Rapidity of staining was accomplished by igniting and burning off, on the slide, the solvent of a small amount of a stain dissolved in a high grade of alcohol. The stains, American made, were generally 1% in 95% alcohol except in the case of acid fuchsin and orange I, where 0.5% was used. Methyl blue, due to low solubility in 95% alcohol, was dissolved in 80% alcohol. When the solubility of a stain was less than 1%, a saturated solution was used. No apparent damage to cells or contents was observed in more than 200 slides stained. The following procedure was followed: Place 2–4 drops of primary stain on slide from absolute alcohol. Ignite and allow solvent to burn off completely. Wash briefly but thoroly in 95% alcohol. Place 4–6 drops of counterstain on slide for 10–20 sec. With woody sections or with stains acting slowly, such as methyl blue and orange G, burn off as with primary stain. Wash in 95% alcohol, clear and examine. To insure proper differentiation, use a 5–10 sec. staining period, with washing and examination repeated until adequately stained, or if sections are 5 mm. or more in length, hold over a white surface until color of section changes to that of the counterstain.

Best combinations for leaf sections proved to be methylene blue with eosin or erythrosin; Victoria green with picric acid; safranin A or rhodamine B with methyl blue. For stem and root sections the best were methylene blue with eosin, erythrosin or orange G; malachite green with acid fuchsin or orange I; and safranin A with methyl blue. A shorter method resulted from using a combination of stains and counterstains in a single solution, burned off as in the previous method. Washing time, generally 5–15 sec., varied with stain combinations and nature of sections. Also such double stains for blood, as Wright's and Giemsa's standardized solutions and Wright's and Jenner's saturated solutions in 95% alcohol, proved satisfactory after ignition. Giemsa's stain should be stopped, i.e., flame blown out, when the glycerin begins to bubble. The following combinations proved satisfactory (numbers following stains indicate volumes in combination): Malachite green, 1, or Victoria green, 1, with acid fuchsin, 10; basic fuchsin, 1, with methyl blue, 10; safranin A, 3, with orange G, 20; methylene blue, 2, with acid fuchsin, 10; methylene blue, 3, with erythrosin, 20. Where cork was present in the section, a triple color combination was obtained with the blood stains and with methylene blue in combination with acid fuchsin, orange G or erythrosin.—*Merritt N. Pope.*

MICROÖRGANISMS

BRYAN, C. S., DEVEREUX, E. D., HIRSCHEY, W. C., and CORBETT, A. C. **The use of brilliant green, sodium azide and dextrose in the microscopic and Hotis tests for streptococcic mastitis.** *North American Veterinarian*, 20, 41–6. 1939.

Experiments were carried out to determine the effectiveness of brilliant green and sodium azide in suppressing bacteria other than streptococci and to determine the value of dextrose and physical factors in obtaining maximum growth of streptococci during the time interval of the microscopic and Hotis tests. (Hotis and Miller, U. S. Dept. Agr. Circ., No. 400, 1936.) From tests on the milk of several hundred cows it was concluded that better results were obtained from a selective preservative containing brilliant green, sodium azide and dextrose than from either preservative used alone and the use of a selective preserva-

tive yielding a final dilution of 1:50,000 of brilliant green, 1:15,000 of sodium azide and 1:1000 of dextrose in the milk is recommended. This preservative increased the accuracy of the microscopic test as compared to the use of brilliant green alone and greatly reduced the time required to read the results by suppressing both the udder micrococci and contaminating bacteria. The use of this preservative in the Hotis test increased the 24-hr. reading from 60 to 81% efficiency and reduced the number of negative cows giving positive tests from 7.3 to 2.6%. Similar improvement was obtained in the 48-hr. reading. The addition of dextrose compensated for the low lactose content of the milk of mastitis-infected cows and permitted better growth of the streptococci in the sample.—*Colin W. Whittaker.* (*Cited from* Chem. Abstr., **34,** 1350. 1940.)

CASTRO, GUILLERMO. **The rapid diagnosis of malaria from thick blood smears.** *J. Lab. & Clin. Med.*, **25,** 1308–10. 1940.

The following modification of the Barber and Kemp method (Pub. Health Rep., **44,** 2330, 1929) is given: Put a drop of blood about 3 times the size necessary for a thin smear on a slide and spread in a circle about ½ inch in diameter made by a wax pencil. Place the slide on a hot plate at 50°–60° C. where it will dry in about one minute. Stain for 3 to 5 min., using a mixture of 3 drops of distilled water and 1 or 2 drops of Giemsa's stain. Wash carefully in a tray of distilled water and dry on a hot plate in an inclined position. The method is rapid, the red cells are completely decolorized, the parasites are deeply and distinctly stained and any basophilic stippling is clearly demonstrated.—*John T. Myers.*

GOULD, S. E. **Incidence of trichinosis among county hospital patients in the Detroit area.** *Amer. J. Clin. Path.*, **10,** 431–59. 1940.

The following two methods are recommended for searching for larvae of *Trichinella spiralis*: (1) Press pectoral muscle and diaphragm between two pieces of plate glass in a metal frame compressed by set screws and examine under a dissecting microscope. (2) Digest these tissues in artificial gastric juice (1% pepsin and 0.7% HCl) 15–24 hr. at 37° C.; allow the chilled digest to settle and wash the sediment. Examine with the microscope. The first method gave 15.4%, the second 17.1%, and a combination of the two 20% positives.—*G. H. Chapman.*

KELLY, CORNELIUS B. **Brilliant green lactose bile and the standard methods completed test in isolation of coliform organisms. A comparative study.** *Amer. J. Pub. Health*, **30,** 1034–9. 1940.

This study was made to determine the efficiency of brilliant green lactose bile broth (Standard Methods formula) as a confirmatory medium for coliform organisms, particularly from shellfish and shellfish-bearing waters. Parallel inoculations were made into this medium and onto EMB agar from all lactose broth tubes which were submitted to the completed test, and results from the two series compared. Comparative tests on 2,087 water samples, of which about 90% were sea waters, indicated that the presence of gas in brilliant green lactose bile broth is a more accurate criterion of the presence of coliforms than the Standard Methods completed test using solid confirmatory media alone. Similar results, but not so marked, were obtained on 2,347 shellfish samples. Definite seasonal variation was observed. Considering all samples, gas production in brilliant green lactose bile broth was obtained with 101.2% of the total coliforms isolated by both methods, while the Standard Methods completed test recovered 93.8%.—*M. W Jennison.*

NABEL, K. **Über die Membran niederer Pilze besonders von Rhizidiomyces bivellatus nov. spez.** *Arch. Mikrob.*, **10,** 515–41. 1939.

The method of Van Wisselingh for identifying chitin and cellulose is adapted for use with membranes of the lower molds by destroying by heat nearly all the other substances present. The procedure is as follows: In a glass tube 6 cm. × 6–7 mm., sealed at one end, place a layer of glycerin 1-2 cm. thick. Introduce the object to be studied into the glycerin, seal off the open end and heat to 300° C. in

an oil bath. Cool, cut off the tube close to the surface of the glycerin, harden the greatly softened tissue in abs. alcohol and wash in dist. water. Transfer tissue to another glass tube containing conc. KOH, seal off and reheat in the oil bath, this time to 180° C.; cool, open, harden in alcohol and wash in dist. water. Bring the material onto a slide and add picric acid. A permanent yellow color indicates chitin while cellulose is shown if the color can be washed out. Add a few drops of I–KI solution and 1% H_2SO_4. Chitosan is shown by a red-violet coloration (Ostwald's color tables No. 10, first part between ga and ia). Treat now with conc. H_2SO_4 and the red-violet color disappears and the chitosan dissolves. If there is no chitin present in the membrane, the tissue remains uncolored after the treatment with I–KI solution and 1% H_2SO_4, but on addition of the conc. H_2SO_4 the presence of cellulose is shown by a clear blue coloration (Ostwald's color table No. 13, first U blue, between ga-ia-la).—*Merritt N. Pope.*

ROE, M. A., LILLIE, R. D., and WILCOX, A. **American azures in the preparation of satisfactory Giemsa stains for malaria parasites.** *Public Health Reports*, **55**, 1272. 1940.

Using a modified Nocht technic, the authors have explored the thiazin dye group in combination with eosin for dyes and proportions giving satisfactory staining of malaria parasites in thin and thick blood films. Methylene blue stains parasite cytoplasm satisfactorily but fails to demonstrate parasite chromatin. Azure B gives clear, sharply defined, purplish red chromatin and sharply defined cytoplasm, a somewhat lighter blue than with methylene blue. Azure A gives denser, deeper reddish purple and less sharply defined chromatin and a grayish lavender parasite cytoplasm which contrasts poorly with red corpuscles or with the background of thick films. The most satisfactory staining was given by mixtures of azure B (1 part) and methylene blue (1 to 4 parts) with the appropriate amount of eosin and with or without a small amount of azure A (0.2 part). Three of these formulae were made up of corresponding quantities of the dry dyes in glycerin and methyl alcohol and gave very satisfactory results when a 1:50 dilution at pH 7.0 for 45 min. was used.

The formulae, in parts by weight, are: (A) Azure A 0.5, azure B 2.0, methylene blue 2.7, eosin Y 5.0, in CP glycerin 50 and CP methyl alcohol 50. (B) Azure B 2.5, methylene blue 2.7, eosin Y 5.0 in the same solvent. (C) Azure B 1.7, methylene blue 3.4, eosin Y 4.9 in the same solvent. These weights are in terms of pure dye and corrections should be made for dye content.

Of these formulae, some observers prefer A, some C. The results are closely similar to those obtained with Grübler's Giemsa solution.—*R. D. Lillie.*

ROGERS, T. HOWARD. **The inhibition of sulphate-reducing bacteria by dyestuffs.** *J. Soc. Chem. Ind.*, **59**, 34–9. 1940.

Bacteria which produce H_2S from inorganic sulfates are of economic importance because of the damage done by the H_2S to materials with which it comes in contact, e.g. corrosion of metals in various industrial conditions. Inhibition of these organisms is often impossible by the use of ordinary disinfectants because they may have corrosive properties. The development of sulfate-reducing bacteria may be inhibited by the use of dyestuffs derived from 3:6-diamino-acridine, such as acriflavine, proflavine, or No. 914 (a flavine dye produced by Imperial Chemical Industries). These dyes are non-corrosive, and are effective in very small quantities. The concentrations of dye required are a function of the degree of contamination; but, as far as it is possible to ascertain from laboratory tests alone, the concentration of dye required in most practical conditions appears to be 1 in 250,000 or less.—*A. P. Bradshaw.*

SCHAEDE, R. **Zum Problem des Vorkommens von chromatischer Substanz bei Bacterien and Actinomycetes.** *Arch. Mikrob.*, **10**, 473–507. 1939.

Study of the chromatic substance of bacteria and Actinomycetes by means of the Fuelgen reaction is reported in detail. Endophytes are fixed in the tissue of the host plant using the fluid of Juel generally (but also Benda's and sublimate in glacial acetic acid) embedded in paraffin and sectioned 5 μ thick. A 6-minute hydrolysis for material fixed in Juel's fluid is satisfactory. A new method for

free-living organisms, which avoids drying on the slide and consequent injury to cell contents, is described as follows: Prepare fixative by adding enough water to dried egg white to make a thick flowing mixture which will "thread" when dropped from the stirring rod. Use camphor as a preservative. In making the preparation mix a small mass of the culture with a drop of 0.8% NaCl solution on a slide. Carry over to a clean slide enough of the mixture for a streak preparation and invert for 2 min. over a dish of an osmic acid fixing fluid containing no acetic acid. Place a small drop of albumin fixative on the slide near the drop of fixed bacteria; mix quickly with the inoculating needle and streak broadly. Before drying can take place, put the slide in 50% alcohol for 10 min. Wash with several drops of 70% alcohol and leave in 95% alcohol for at least 24 hr. Treat by the method of Fuelgen, hydrolizing 8 min.—*Merritt N. Pope.*

SOYANO, Y. **A new technique: "mica method" for vital observation.** (In Japanese with English resume). *Botany and Zoology: Theoretical and Applied* (Tokyo), **8**, 106. 1940.

To overcome the difficulty of focusing thru the depth of a drop in the case of hanging-drop preparations, the author touches a clear mica flake to the drop. Because the flake is very light, surface tension holds it close beneath the cover slip, spreading the drop thin and permitting ready focusing.—*J. L. Mohr.*